

**Dynamics of symbiont populations in the facultative  
chemosymbioses of thyasirid bivalves**

by

© Jason R. Laurich

A thesis submitted to the

School of Graduate Studies

in partial fulfillment of the requirements for the degree of

**Masters of Science**

Department of Biology

Memorial University of Newfoundland

**June 2014**

St. John's

Newfoundland

## Abstract

Within the family Thyasiridae, symbioses between hosts and sulphur-oxidizing bacteria are not present in all species. Bacteria that associate with thyasirids are extracellular, digested by host cells, and presumed to be facultatively symbiotic. Here, the dynamic and unstable nature of thyasirid-symbiont associations is characterized. We first describe a *Thyasira* cf. *gouldi* cryptic species complex in which some species possess symbionts, while others have lost them. Within symbiotic *T.* cf. *gouldi* species, both symbiont abundance and the nutritional importance of symbiont-derived nutrients fluctuate over yearly cycles. *T.* cf. *gouldi* symbionts are environmentally acquired, and association with host bivalves appears to result in the loss of both flagella and magnetosome chains. Finally, we show that hosts may control symbiont populations by mediating their access to reduced sulphur: exposing thyasirids to a constant source of reduced sulphur results in increased bacterial division which can result in host cell overgrowth and host mortality.

## **Acknowledgments**

First and foremost, I would like to acknowledge the support of Dr. Suzanne C. Dufour, who presented me with the opportunity to conduct this Master's thesis research. I am exceptionally grateful for her enthusiasm, her support and her patience. She helped provide me with direction in terms of my research goals and the identification of productive research questions. Additionally, I am grateful for her financial assistance through her Natural Sciences and Engineering Research Council of Canada Discovery Grant. I must also thank NSERC for my own personal funding in the form of an NSERC CGS (Canadian Graduate Studies) M award, which I held over the course of my first year of graduate studies, as well as the French government for granting me a CBIE/ French Government award, allowing me to spend four months at the Université de Bretagne Occidentale over the summer of 2013. The time spent there under the supervision of Dr. Christine Paillard at the Institut Universitaire Européen de la Mer was invaluable in my training as a graduate student, and produced many interesting results and findings that formed an integral portion of my master's thesis.

I would also like to thank several people who played important supportive roles in the completion of my research. Bob Hooper and staff of the Bonne Bay Marine Station were ever accommodating, and provided immense assistance in the collection of thysirids from Bonne Bay, Newfoundland. I would also like to thank Stephanie Tucker and Kate Williams of the Electron Microscopy/ Flow Cytometry unit at the Health Sciences Center for their excellent assistance in the preparation of samples for transmission electron analysis and subsequent examination. Finally, I would like to thank

the members of my supervisory committee, Drs. Andrew Lang and Brian Staveley, for valuable input on the direction of my master's research, and all of the friends and family who provided invaluable support over the course of my graduate research project.

## Table of Contents

Abstract .....	ii
Acknowledgements .....	iii
Table of Contents .....	v
List of Tables .....	viii
List of Figures .....	ix
List of Abbreviations and Symbols .....	x
List of Appendices .....	xi
Introduction .....	1
<i>Chemosymbiosis and the family Thyasiridae</i> .....	1
<i>Thyasira cf. gouldi cryptic species complex and loss of symbiosis</i> .....	3
<i>Mixotrophy and nutritional flexibility</i> .....	4
<i>Symbionts of thyasirid bivalves</i> .....	6
<i>Maintenance of stability in chemosymbiotic mutualisms</i> .....	8
<i>Works Cited</i> .....	9
Overview: Summary of Goals and Objectives .....	15
Co-Authorship Statement .....	16
Chapter 1: Seasonal variation in symbiont abundance in the thyasirid bivalve <i>Thyasira</i> cf. <i>gouldi</i> .....	18

Abstract .....	18
Introduction .....	19
Materials and Methods .....	22
<i>Sample collection</i> .....	22
<i>Gill fixation and transmission electron microscopy</i> .....	24
<i>Quantitative analysis</i> .....	24
<i>Statistical analysis</i> .....	28
<i>Generalized additive model construction</i> .....	29
Results .....	30
<i>Temporal variability in symbiont abundance</i> .....	30
<i>Factors influencing symbiont abundance and membrane whorl density</i> .....	31
<i>Symbiont abundance and membrane whorl density: generalized additive models</i> .....	33
Discussion .....	36
Conclusions .....	40
Works Cited .....	41
Chapter 2: Manipulating life and death in facultative chemosymbioses; control of bacterial population dynamics in the Thyasiridae .....	45
Abstract .....	45
Introduction .....	46

Materials and Methods.....	51
<i>Sample collection</i> .....	51
<i>Experimental design</i> .....	52
<i>Transmission electron microscopy</i> .....	54
Results.....	56
Thyasira flexuosa.....	58
Thyasira cf. gouldi .....	60
<i>Death of bacteriocytes and host mortality</i> .....	62
Discussion .....	65
<i>Division of bacterial symbionts</i> .....	65
<i>Thiosulphate exposure and symbiont division</i> .....	68
Conclusions.....	76
Works Cited .....	78
Thesis Summary.....	84
<i>Summary of results</i> .....	84
<i>Conclusions</i> .....	86
<i>Future directions</i> .....	89
<i>Works Cited</i> .....	90
Appendices.....	93

## List of Tables

Table 1.1: Number of symbiotic <i>Thyasira</i> cf. <i>gouldi</i> specimens used for quantitative and statistical analysis over the course of the 2011 and 2012 sampling seasons, from each of the three sampling sites. ....	24
Table 1.2: Model selection for the influence of time on bacterial abundance. 3 generalized additive models (1-3), with smoothing functions “s()” applied to the continuous effect of time, and one linear model (4) were considered. The incorporation of interactive effects between terms is indicated by a “*”. Relevant p-values and Aikake Information Criterion (AIC) scores are presented. ....	34
Table 2.1: Number of thyasirids examined under the transmission electron microscope for the experiments on both species at various elapsed times. Sample sizes are listed for control specimens (maintained in filtered seawater) and experimental specimens kept in the presence of dissolved thiosulphate (TS). The examination of deceased individuals is indicated by the presence of an asterisk (*) after the sample size .....	55



## List of Figures

Figure 1.1: Map showing the location of the three sampling locations in Bonne Bay .....	23
Figure 1.2: Transmission electron micrographs of a <i>Thyasira</i> cf. <i>gouldi</i> gill filament .....	27
Figure 1.3: Transmission electron micrographs of <i>Thyasira</i> cf. <i>gouldi</i> gill filaments.....	31
Figure 1.4: (A) Variation in symbiont abundance over the 8 sample collection dates .....	33
Figure 1.5: Generalized additive model for variation in symbiont abundance .....	35
Figure 2.1: Transmission electron micrographs of <i>Thyasira flexuosa</i> and <i>T.</i> cf. <i>gouldi</i> .....	57
Figure 2.2: Transmission electron micrographs of <i>Thyasira flexuosa</i> .....	59
Figure 2.3: Transmission electron micrographs of <i>Thyasira</i> cf. <i>gouldi</i> .....	60
Figure 2.4: Transmission electron micrographs of ruptured <i>Thyasira flexuosa</i> .....	64

## List of Abbreviations and Symbols

In alphabetical order:

**μm** : Micrometer  
**AIC** : Akaike information criterion  
**B<sub>A</sub>** : Area occupied by bacterial symbionts  
**C** : Celsius  
**cm** : Centimeter  
**d** : day  
**EC<sub>A</sub>** : Extracellular area  
**g** : Gram  
**h** : Hour  
**GAM** : Generalized additive model  
**IC<sub>A</sub>** : Intracellular area  
**m** : Meter  
**M** : Molar  
**mm** : Millimeter  
**MW<sub>A</sub>** : Area occupied by membrane whorls  
**MW<sub>D</sub>** : Membrane whorl density  
**NL** : Newfoundland and Labrador  
**nm** : Nanometer  
**OTU** : Operational taxonomic unit  
**S<sub>Abd</sub>** : Symbiont abundance  
**T<sub>A</sub>** : Total cell area  
**TEM** : Transmission electron microscopy  
**TS** : Thiosulphate

## List of Appendices

Appendix 1: Batstone, R. T., Laurich, J. R., Salvo, F., & Dufour, S. C. (2014). Divergent chemosymbiosis-related characters in <i>Thyasira</i> cf. <i>gouldi</i> (Bivalvia: Thyasiridae). <i>PLoS ONE</i> .9:3, e92856.....	93
Appendix 2: Dufour, S. C., Laurich, J. R., Batstone, R. T., McCuaig, B., Elliot, A., & Poduska, K. M. (2014). Magnetosome-containing bacteria living as symbionts of bivalves. <i>The ISME Journal</i> .doi: 10.1038/ismej.2014.93.....	103

## **Introduction**

### **Chemosymbiosis and the family Thyasiridae**

Symbioses, defined as intimate associations between two different species (Roeselers & Newton, 2012) are common in the Bivalvia (Mollusca). In this group, a remarkable diversity of host organisms form associations with chemosynthetic bacterial symbionts capable of the fixation of inorganic carbon driven by the oxidation of reduced chemicals (Taylor & Glover, 2006). Seven bivalve families are currently known to associate with chemoautotrophic bacteria; the Solemyidae (Krueger *et al.*, 1996; Stewart & Cavanaugh, 2006a), the Vesicomyidae (Fisher & Childress, 1986; Childress *et al.*, 1991), the Mytilidae (Southward, 2008), the Lucinidae (Diouris *et al.*, 1988; Williams *et al.*, 2004), the Nucinellidae (Oliver & Taylor, 2012), the Montacutidae (Oliver *et al.*, 2013) and the Thyasiridae (Dando & Southward, 1986; Dufour, 2005). Within these diverse families there exists considerable variation in crucial parameters of the symbiotic association, including relative reliance on symbiotically-derived organic carbon, the method of symbiont transmission, as well as the morphological adaptations of the host and the localization of bacterial symbionts in host gill epithelial cells (Roeselers & Newton, 2012).

The Thyasiridae are one of the most diverse and widespread families of chemosymbiotic bivalves (Taylor *et al.*, 2007), colonizing a variety of cold water environments such as oil fields (Oliver & Kileen, 2002), cold seeps (Oliver & Sellanes, 2005; Carlier *et al.*, 2010), hydrothermal vents (Southward *et al.*, 2001), and organically enriched or polluted sediments (Dando & Southward, 1986; Dando & Spiro, 1993).

Within this family, there exists an unusual degree of variation in the association of host species with chemoautotrophic bacterial symbionts. Unlike other chemosymbiotic bivalve families such as the Lucinidae, in which all known species associate with sulphur-oxidizing bacterial symbionts (Taylor & Glover, 2006), not all members of the Thyasiridae harbour chemosynthetic bacterial symbionts. Within the single genus *Thyasira*, some species, such as *Thyasira ferruginea* lack bacterial symbionts altogether (Dando & Southward, 1986), while species such as *T. flexuosa*, *T. sarsi*, *T. gouldi*, and *T. equalis* harbour bacterial symbionts in their gill epithelia (Reid & Brand, 1986; Dando & Southward, 1986; Diouris *et al.*, 1988; Batstone *et al.*, 2014). The sole broad-scale investigation of host morphological adaptations to the possession of bacterial symbionts further underscores this level of intra-familial variation, with 15 of 26 species examined lacking bacterial symbionts altogether (Dufour, 2005). Variation in the possession of bacterial symbionts in the Thyasiridae is linked to extensive inter-specific discrepancies in gill filament morphology. Dufour (2005) has identified three different gill types in the Thyasiridae distinguished by the degree of abfrontal expansion of the gill filaments, a key adaptation permitting hosts to provide greater surface area for bacterial colonization (Reid & Brand, 1986). Most symbiotic thyasirids display type 3 gill filament morphology, characterized by extensive expansion of the subfilamentar gill epithelia to form a bacteriocyte zone in which symbionts are housed, while asymbiotic thyasirids are distinguished by a lack of abfrontal expansion of the gill filaments (type 1 and 2 gills, Dufour, 2005).

Furthermore, the Thyasiridae are distinct from other groups of chemosymbiotic bivalves in several other important aspects. With the exception of *Maorithyas hadalis*, the

symbionts of thyasirids are housed extracellularly, in enlarged pockets delineated by extensions of the host cytoplasm and cell membrane (Fujiwara *et al.*, 2001; Dufour, 2005). In addition to the extracellular localization of symbionts, sulphur-oxidizing bacterial symbionts are significantly smaller than symbionts seen in other families such as the Lucinidae (Le Pennec *et al.*, 1988b). Additionally, the symbionts of thyasirid bivalves display a greater degree of phylogenetic diversity and lack of conserved symbiont identity between different host species, with multiple independent acquisitions of symbionts in distinct thyasirid lineages (Taylor *et al.*, 2007; Rodrigues & Duperron, 2011). Due to the high degree of intra-familial variation in the possession of bacterial symbionts (Dando & Southward, 1986; Dufour, 2005), as well as the extracellular location of bacterial symbionts and lack of conserved symbiont identity within the family (Dufour, 2005; Rodrigues & Duperron, 2011), the Thyasiridae are thought to represent one of the least derived chemosymbioses in the Bivalvia (Roeselers & Newton, 2012).

### ***Thyasira* cf. *gouldi* cryptic species complex and loss of symbiosis**

The recent discovery of a cryptic *Thyasira* cf. *gouldi* species complex further illustrates the incredible degree of flexibility, inter-specific variation in the possession of bacterial symbionts and potential for evolutionary instability of chemosymbiotic associations in the Thyasiridae. In a paper to which I contributed significantly, Batstone *et al.* (2014) describe the discovery of a cryptic *T.* cf. *gouldi* species that includes both symbiotic and asymbiotic host OTUs (Operational Taxonomic Units). Symbiont presence is associated with the abfrontal expansion of gill filaments (gill type 3, OTUs 1 and 2) and the subsequent increase in gill epithelial surface area for the hosting of bacterial symbionts (Batstone *et al.*, 2014), as described in Dufour (2005). In contrast, asymbiotic

individuals (OTU 3) possess translucent, thin gills lacking abfrontal expansion of the filaments (Batsone *et al.*, 2014). The ancestral state of the original Bonne Bay *T. cf. gouldi* population is postulated to be characterized by a symbiotic association with sulphur-oxidizing bacteria. As such, the Thyasiridae not only represent a relatively early stage in the evolution of complex chemosymbioses (Roeselers & Newton, 2012), but are also characterized by the potential for rapid evolutionary loss of symbiosis, leading to fine-scale phylogenetic and geographic patchiness in the possession of bacterial symbionts (Batstone *et al.*, 2014).

### **Mixotrophy and nutritional flexibility**

In addition to extensive variation in the possession of bacterial symbionts in the Thyasiridae, symbiotic members of this family are distinguished by the facultative nature of the symbiotic associations, with symbiotically derived nutrition likely serving as a supplement to particulate feeding (Dufour, 2005). Unlike other obligate associations in which hosts lack digestive systems and are completely reliant upon symbiotic carbon fixation (Stewart & Cavanaugh, 2006b; Nyholm *et al.*, 2012), symbiotic thyasirids are mixotrophic. Though reduced, symbiotic thyasirids retain structures associated with heterotrophic feeding in bivalves, such as the labial palps and digestive gland (Allen, 1958; Donval *et al.*, 1989). The relative importance of symbiotic and heterotrophic carbon sources to the host diet is subject to notable fluctuations based on a number of environmental factors. Changes in sediment sulphide concentrations have previously been correlated with transition from up to 76% reliance on symbiotic carbon to an almost completely heterotrophic lifestyle in *Thyasira sarsi* (Dando & Spiro, 1993). Additionally, authors have postulated a greater reliance on symbiotically derived organic carbon over

the winter months, as evidenced by variation in the activity of the digestive gland in *Thyasira flexuosa* (Donval *et al.*, 1989), and laboratory experiments have indicated that symbiont abundance in thyasirids is related to particulate food and sulphide availability (Dufour & Felbeck, 2006). Nonetheless, the possibility that symbiont abundance, along with the relative nutritional importance of symbiotic carbon, varies over temporal cycles has never been rigorously examined in the Thyasiridae.

Thyasirids frequently inhabit organically enriched, shallow sediments receiving large amounts of organic matter input through a variety of methods, including sewage discharge, river runoff and terrestrial export of plant detritus, and seasonal phytoplankton blooms (Dando & Southward, 1986; Dando & Spiro, 1993; Diaz & Rosenberg, 2001). Increases in organic matter content in benthic environments stimulate the activities of sulphate-reducing bacteria, leading to oxygen depletion and sulphide accumulation (Westrich & Berner, 1984; Faulwetter *et al.*, 2013). Thyasirids are common in such environments, and are postulated to be ecological engineers, due to their capacity to oxygenate sediments and remove accumulated sulphides through their bioirrigation and the construction of pedal tracts involved in sulphide mining (Dando & Spiro, 1993; Dufour & Felbeck, 2003; Dando *et al.*, 2004). In conjunction with temporal cycles in vertical organic matter export to the benthos, the ability of thyasirid bivalves to deplete sedimentary reserves of reduced sulphur compounds may exert a profound influence on the availability of sulphides in organically enriched sediments. This makes the Thyasiridae an excellent model group for studying natural influences affecting reliance on symbiotic carbon in facultative chemosymbioses and understanding which factors surround the evolutionary impact of chemosymbiosis. In Chapter 1, I explore the



possibility of seasonal variation in the abundance of bacterial symbionts in the gill epithelia of *Thyasira* cf. *gouldi* in the interest of exploring the degree of nutritional flexibility and temporal variability in symbiont population size in this family of chemosymbiotic bivalves.

### **Symbionts of thyasirid bivalves**

Like all known bacterial symbionts of chemoautotrophic bivalves, the symbionts of thyasirids fall within the gammaproteobacteria, forming a clade with the symbionts of other chemosymbiotic organisms such as lucinids and *Riftia pachyptila* (Distel & Cavanaugh, 1994; Dubilier *et al.*, 2008; Dufour *et al.*, 2014). Symbionts are held in extracellular pockets defined by extensions of the host cell membrane and cytoplasm, and bacteria are periodically endocytosed to undergo lysosomal degradation, providing nutrients directly to the host cell (Southward, 1986; Le Pennec *et al.*, 1988a; Dufour, 2005). Thyasirid symbionts are thought to be facultative and environmentally acquired, as evidenced by their extracellular location, along with the diversity of bacterial symbionts between closely related thyasirid species and the potential for host clams of a single species to associate with different symbiont phylotypes (Dufour, 2005; Rodrigues & Duperron, 2011; Duperron *et al.*, 2012). Environmental acquisition raises a suite of challenges for the maintenance of evolutionary stable mutualisms. Hosts must be able to locate their symbionts and discriminate between mutualistic and potentially exploitative bacterial phylotypes in order to assure repeated mutualistic interactions between hosts and symbionts across generations (Douglas, 2008; Chaston & Goodrich-Blair, 2010). In turn, free-living forms of the bacterial symbionts must be capable of living in both the tissues of their facultative hosts and in the environment. Unlike vertically transmitted symbionts,

which may lose genes as vital as those responsible in processes such as cytokinesis, motility, and heterotrophy through co-evolution and the loss of symbiont independence (Kuwahara *et al.*, 2007), environmentally acquired symbionts must retain the metabolic and physiological flexibility that allows them to navigate steep chemical gradients and support their metabolic needs without the provisioning of substrates by their host organism (Stewart & Cavanuagh, 2006b; Robidart *et al.*, 2008).

In thyasirids, bacterial symbionts, similar to the case observed for the facultative symbionts of *Riftia pachyptila* (Robidart *et al.*, 2008), retain prominent adaptations to a free-living stage in reduced, sulphidic sediments. A recent paper published by Dufour *et al.* (2014) to which I made substantial contributions, documents the discovery of magnetosomes, protein-bound biomineralized iron, within the symbionts of *Thyasira* cf. *gouldi*. In sulphur-oxidizing microaerophilic bacteria such as the free-living stage of thyasirid symbionts, magnetosome chains produce a magnetic dipole within the cell, allowing these chemosynthetic microaerophiles to locate the oxic-anoxic interface in reduced sediments, where bacteria have access to both oxygen and the reduced chemical compounds required to sustain their metabolism (Lefèvre & Bazylinski, 2013). Symbionts appear to retain chains of these magnetic particles when not associated with host bacteriocytes, while the adoption of a symbiotic existence results in the loss of integrity (and hence, functionality) of magnetosome chains, along with the postulated loss of other free-living physiological adaptations such as flagella (Dufour *et al.*, 2014). The burrowing and sulphide mining behaviours of thyasirids likely produce oxic-anoxic interfaces along burrows and pedal tracts, thus allowing them to cultivate magnetotactic, sulphur-oxidizing bacteria in nearby sediments and assist them in the environmental acquisition of

free-living bacteria, which may otherwise be scarce in sediments (Dando *et al.*, 2004; Hakonen *et al.*, 2010; Dufour *et al.*, 2014).

### **Maintenance of stability in chemosymbiotic mutualisms**

Following the environmental acquisition of symbionts from the surrounding sediments, host organisms must be able to derive tangible fitness benefits from their association with chemosynthetic bacterial symbionts. In thyasirids, nutrient transfer occurs through direct lysosomal digestion (Southward, 1986; Le Pennec *et al.*, 1988a), and hosts are often incredibly reliant on the digestion of bacterial symbionts, which in some cases can contribute upwards of 50% of the hosts' nutritional input (Spiro *et al.*, 1986; Dando & Spiro, 1993). In turn, thyasirids supply their symbionts with a steady supply of reduced sulphur compounds, through either the transport of reduced compounds in the haemolymph of thyasirids' feet or through the direct uptake of sulphidic porewater through ventilation (Dufour & Felbeck, 2003; Dando *et al.*, 2004). This elevated supply of reduced sulphur compounds provides the basis for the exceptional metabolic activity of bacterial symbionts (Caro *et al.*, 2009). However, it also underscores the importance of host control over bacterial symbiont population dynamics, lest enrichment of the symbionts' medium result in unrestrained proliferation and the overgrowth of host tissues (Neckelmann & Muscatine, 1983). While the mechanisms of host control over symbiont population dynamics are unknown in the Thyasiridae, other groups such as the vesicomysids have restricted bacterial division in their vertically transmitted symbionts through co-evolution and the subsequent loss of crucial symbiotic genes relating to cell division in bacteria (Kuwahara *et al.*, 2007). In environmentally transmitted symbioses characterized by a lack of co-evolution between host and symbiont, hosts such as the

lucinid bivalves are able to suppress bacterial division through the production of bacteriostatic compounds (Caro *et al.*, 2007; Caro *et al.*, 2009). *Riftia pachyptila* symbiont populations are regulated by tightly controlled cycles of host cell differentiation, resulting in eventual apoptosis coupled with the elimination of symbionts in degenerating bacteriocytes (Pflugfelder *et al.*, 2009). As the Thyasiridae represent an early stage in the evolution of complex chemosymbioses (Roeselers & Newton, 2012), they may lack such complex mechanisms of host control over bacterial symbiont population dynamics. In Chapter 2, I investigate the effect of thiosulphate enrichment on the symbiont populations of thyasirid bivalves in order to understand the nature of population control in this family and evaluate the potential importance of behavioural adaptations involving host control over the supply of reduced sulphur compounds through sulphide mining and burrow ventilation behaviours.

## Works Cited

- Allen, J. (1958). On the basic form and adaptations to habitat in the Lucinacea (Eulamellibranchia). *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences*. 241, 421-484.
- Batstone, R. T., Laurich, J. R., Salvo, F., & Dufour, S. C. (2014). Divergent chemosymbiosis-related characters in *Thyasira* cf. *gouldi* (Bivalvia: Thyasiridae). *PLoS ONE*. 9:3, e92856.
- Carlier, A., Bénédicte, R., Rodrigues, C. F., Sarrazin, J., Karine O., Grall, J., & Clavier, J. (2010). Heterogeneous energetic pathways and carbon sources on deep eastern Mediterranean cold seep communities. *Marine Biology*. 157:11, 2545-2565.
- Caro, A., Gros, O., Got, P., De Wit, R., & Troussellier, M. (2007). Characterization of the population of the sulfur-oxidizing symbiont of *Codakia orbicularis* (Bivalvia, Lucinidae) by single-cell analyses. *Applied and Environmental Microbiology*. 73:7, 2101-2109.

- Caro, A., Got, P., Bouvy, M., Troussellier, M., & Gros, O. (2009). Effects of long-term starvation on a host bivalve (*Codakia orbicularis*, Lucinidae) and its symbiont population. *Applied and Environmental Microbiology*. 75:10, 3304-3313.
- Chaston, J., & Goodrich-Blair, H. (2010). Common trends in mutualism revealed by model associations between invertebrates and bacteria. *FEMS Microbiology Reviews*. 34, 41-58.
- Childress, J. J., Fisher, C. R., Favuzzi, J. A., & Sanders, N. K. (1991). Sulfide and carbon dioxide uptake by the hydrothermal vent clam, *Calyptogena magnifica*, and its chemoautotrophic symbionts. *Physiological Zoology*. 64:6, 1444-1470.
- Dando, P. R., & Southward, A. J. (1986). Chemoautotrophy in bivalve molluscs of the genus *Thyasira*. *Journal of the Marine Biology Association of the United Kingdom*. 66, 915-929.
- Dando, P. R., Southward A. J., & Southward, E. C. (2004). Rates of sediment sulphide oxidation by the bivalve mollusc *Thyasira sarsi*. *Marine Ecology Progress Series*. 280, 181-187.
- Dando, P. R., & Spiro, B. (1993). Varying nutritional dependence of the thyasirid bivalves *Thyasira sarsi* and *T. equalis* on chemoautotrophic symbiotic bacteria, demonstrated by isotope ratios of tissue carbon and shell carbonate. *Marine Ecology Progress Series*. 92, 151-158.
- Diaz, R. J., & Rosenberg, R. (2001). Overview of anthropogenically-induced hypoxic effects on marine benthic fauna. In N. N. Rabalais, & R. E. Turner (Eds.), *Coastal Hypoxia: Consequences for living resources and ecosystems*. 129-145. Washington, D.C.
- Diouris, M., Moraga, D., Le Pennec, M., Herry, A., & Donval, A. (1988). Chimioautotrophie et nutrition chez les Lucinacea, bivalves littoraux de milieux réducteurs. I: Activités enzymatiques des bactéries chimioautotrophes associées aux branchies. *Halietis*. 18, 195-205.
- Distel, D. L., & Cavanaugh, C. M. (1994). Independent phylogenetic origins of methanotrophic and chemoautotrophic bacterial endosymbiosis in marine bivalves. *Journal of Bacteriology*. 176, 1932-1938.
- Donval, A., Le Pennec, M., Herry, A., & Diouris, M. (1989). Nutritional adaptations of littoral bivalve molluscs to reducing biotopes. In J. S. Ryland, & P. A. Tyler (Eds.), *Reproduction, Genetics and Distributions of Marine Organisms. Proceedings of the 23rd European Marine Biology Symposium*. 373-378. Swansea.

- Douglas, A.E. (2008). Conflict, cheats and the persistence of symbioses. *New Phytologist*. 177, 849-858.
- Dubilier, N, Bergin, C., & Lott, C. (2008). Symbiotic diversity in marine animals: the art of harnessing chemosynthesis. *Nature Reviews Microbiology*. 6:10, 725-740.
- Dufour, S. C. (2005). Gill anatomy and the evolution of symbiosis in the bivalve family Thyasiridae. *Biological Bulletin*. 208:3, 200-212.
- Dufour, S.C., & Felbeck, H. (2003). Sulphide mining by the superextensile foot of symbiotic thyasirid bivalves. *Nature*. 426:6962, 65-67.
- Dufour, S. C., & Felbeck, H. (2006). Symbiont abundance in thyasirids (Bivalvia) is related to particulate food and sulphide availability. *Marine Ecology Progress Series*. 320, 185-194.
- Dufour, S. C., Laurich, J. R., Batstone, R. T., McCuaig, B., Elliot, A., & Poduska, K. M. (2014). Magnetosome-containing bacteria living as symbionts of bivalves. *The ISME Journal*. doi:10.1038/ismej.2014.93.
- Duperron, S., Gaudron, S.M., Rodrigues C.F., Cunha M.R., Decker C, & Olu, K. (2012). An overview of chemosynthetic symbioses in bivalves from the North Atlantic and Mediterranean Sea. *Biogeosciences Discussions*. 9, 16815-16875.
- Faulwetter, J.L., Burr, M.D., Parker, A.E., Stein, O. R., & Camper, A.K. (2013). Influence of season and plant species on the abundance and diversity of sulfate reducing bacteria and ammonia oxidizing bacteria in constructed wetland microcosms. *Microbial Ecology*. 65:1, 11-127.
- Fisher, C. R., & Childress, J. J. (1986). Translocation of fixed carbon from symbiotic bacteria to host tissues in the gutless bivalve *Solemya reidi*. *Marine Biology*. 93:1, 59-68.
- Fujiwara, Y., Kato, C., Masui, N., Fujikura, K., & Kojima, S. (2001). Dual symbiosis in the cold-seep thyasirid clam *Maorithyas hadalis* from the hadal zone in the Japan trench, Western Pacific. *Marine Ecology Progress Series*. 214, 151-159.
- Hakonen, A., Hulth, S., & Dufour, S. (2010). Analytical performance during radiometric long-term imaging of pH in bioturbated sediments. *Talanta*. 81, 1393-1401.
- Lefèvre C. T., & Bazylinski, D. A. (2013). Ecology, diversity, and evolution of magnetotactic bacteria. *Microbiology and Molecular Biology Reviews*. 77:3, 497-526.

- Le Pennec, M., Diouris, M., & Herry, A. (1988a). Endocytosis and lysis of bacteria in gill epithelium of *Bathymodiolus thermophilus*, *Thyasira flexuosa*, and *Lucinella divaricata* (Bivalve, Molluscs). *Journal of Shellfish Research*. 7:3, 483-489.
- Le Pennec, M., Herry, A., Diouris, M., Moraga, D., & Donval, A. (1988b). Chimioautotrophie chez les Lucinacea, bivalves littoraux de milieux réducteurs. II- Caractéristiques morphologiques des bactéries symbiotiques et modifications structurelles adaptives des branchies de l'hôte. *Haliotis*. 18: 207-217.
- Neckelmann, N., & Muscatine, L. (1983). Regulatory mechanisms maintaining the *Hydra-Chlorella* symbiosis. *Proceedings of the Royal Society of London. Series B, Biological Sciences*. 219, 193-210.
- Nyholm, S.V., Song, P., Dang, J., Bruce, C., & Girguis, P.R. (2012). Expression and putative function of innate immunity genes under *in situ* conditions in the symbiotic hydrothermal vent tubeworm *Ridgeia piscesae*. *PLoS ONE*. 7:6, e38267.
- Krueger, D. M., Gustafson, R.G., & Cavanaugh, C. M. (1996). Vertical transmission of chemoautotrophic symbionts in the bivalve *Solemya velum* (Bivalvia: Protobranchia). *Biological Bulletin*. 190:2, 195-202.
- Kuwahara, H., Yoshida, T., Yoshihiro, T., Shimamura, S., Nishi, S., Harada, M., Matsuyama, K., Takishita, M., Kawato, M., Uematsu, K., Fujiwara, Y., Sato, T., Kato, C., Kitagawa, M., Kato, I., & Maruyama, T. (2007). Reduced genome of the thioautotrophic intracellular symbiont in a deep-sea clam, *Calyptogena okutanii*. *Current Biology*. 17, 881-886.
- Oliver, P. G., & Kileen, I. J. (2002). The Thyasiridae (Mollusca: Bivalvia) of the British continental shelf and North Sea oil fields: an identification manual. *Wales: Studies in Marine Biodiversity and Systematics from the National Museum of Wales. BIOMOR Reports*. 3, 1-73.
- Oliver, P. G., & Sellanes, J. (2005). New species of Thyasiridae from a methane seepage area off Concepción, Chile. *Zootaxa*. 1092, 1-20.
- Oliver, P. G., Southward, E. C., & Dando, P. R. (2013). Bacterial symbiosis in *Syssitomya pourtalesiana* Oliver, 2012 (Galeommatoidea: Montacutidae), a bivalve commensal with the deep-sea echinoid *Pourtalesia*. *Journal of Molluscan Studies*. 79:1, 30-41.
- Oliver, P. G., & Taylor, J. D. (2012). Bacterial symbiosis in the Nucinelidae (Bivalvia: Solemyida) with descriptions of two new species. *Journal of Molluscan Studies*. 78:1, 81-91.

- Pflugfelder, B., Cary, S. C., & Bright, M. (2009). Dynamics of cell proliferation and apoptosis reflect different life strategies in hydrothermal vent and cold seep vestimentiferan tubeworms. *Cell and Tissue Research*. 337, 149-165.
- Reid, R. G. B., & Brand, D. G. (1986). Sulfide-oxidizing symbiosis in Lucinaceans: implications for bivalve evolution. *The Veliger*. 29:1, 3-24.
- Robidart, J.C., Bench, S.R., Feldman, R.A., Novoradovsky, A., Podell, S.B., Gaasterland, T., Allen, E. E., & Felbeck, H. (2008). Metabolic versatility of the *Riftia pachyptila* endosymbiont revealed through metagenomics. *Environmental Microbiology*. 10:3, 727-737.
- Rodrigues, C. F., & Duperron, S. (2011). Distinct symbiont lineages in three thyasirid species (Bivalvia: Thyasiridae) from the eastern Atlantic and Mediterranean Sea. *Naturwissenschaften*. 98:4, 281-287.
- Roeselers, G., & Newton, I. L. G. (2012). On the evolutionary ecology of symbioses between chemosynthetic bacteria and bivalves. *Applied Microbiology and Biotechnology*. 94:1, 1-10.
- Southward, E.C. (1986). Gill symbionts in thyasirids and other bivalve molluscs. *Journal of the Marine Biology Association of the United Kingdom*. 66, 889-914.
- Southward, E. C. (2008). The morphology of bacterial symbioses in the gills of mussels of the genera *Adipicola* and *Idas* (Bivalvia: Mytilidae). *Journal of Shellfish Research*. 27:1, 139-146.
- Southward, E. C., Gebruk, A., Kennedy, H., Southward, A. J., & Chevaldonné, P. (2001). Different energy sources for three symbiont-dependent bivalve molluscs at the Logatchev hydrothermal site (Mid-Atlantic Ridge). *Journal of the Marine Biology Association of the United Kingdom*. 81:4, 655-661.
- Spiro, B., Greenwood, P.B., Southward, A. J., & Dando, P.R. (1986).  $^{13}\text{C}/^{12}\text{C}$  ratios in marine invertebrates from reducing sediments: confirmation of nutritional importance of chemoautotrophic endosymbiotic bacteria. *Marine Ecology Progress Series*. 28, 233-240.
- Stewart, F. J., & Cavanaugh, C. M. (2006a). Bacterial endosymbioses in *Solemya* (Mollusca: Bivalvia)- model systems for studies of symbiont-host adaptation. *Antonie Van Leeuwenhoek*. 90:4, 343-360.
- Stewart, F. J., & Cavanaugh, C. M. (2006b). Symbiosis of thioautotrophic bacteria with *Riftia pachyptila*. *Progress in Molecular and Subcellular Biology*. 41, 197-225.



- Taylor, J. D., & Glover, E. A. (2006). Lucinidae (Bivalvia)- the most diverse group of chemosymbiotic molluscs. *Zoological Journal of the Linnean Society*. 148:3, 421-438.
- Taylor, J. D., Williams S. T., & Glover, E. A. (2007). Evolutionary relationships of the bivalve family Thyasiridae (Mollusca: Bivalvia), monophyly and superfamily status. *Journal of the Marine Biology Association of the United Kingdom*. 87, 565-574.
- Westrich, J. T., & Berner, R. A. (1984). The role of sedimentary organic matter in bacterial sulfate reduction: the G model tested. *Limnology and Oceanography*. 29:2, 236-249.
- Williams, S. T., Taylor, J. D., & Glover, E. A. (2004). Molecular phylogeny of the Lucinoidea (Bivalvia): non-monophyly and separate acquisition of bacterial chemosymbiosis. *Journal of Molluscan Studies*. 70:2, 187-202.

## **Overview: Summary of Goals and Objectives**

In this Master's thesis, I summarize the results of my studies on the dynamic nature of symbiotic associations between sulphur-oxidizing chemosynthetic bacteria and thyasirid bivalves. Aspects of my research relating to inter-specific variation in the possession of bacterial symbionts within a cryptic *Thyasira* cf. *gouldi* species complex, as well the physiology of facultative bacterial symbionts associated with environmental acquisition by the host were incorporated into two research papers published by the Dufour lab (Batstone *et al.*, 2014; Dufour *et al.*, 2014). These two papers, though not constituting formal chapters of my thesis, are included as appendices.

The first primary goal of my research was to investigate the possibility of temporal, seasonally-induced fluctuations in the abundance and importance of bacterial symbionts in *T. cf. gouldi* (Chapter 1). Additionally, I conducted experiments designed to evaluate whether or not symbiont division occurs within thyasirid bacteriocytes and the mechanisms of host control over bacterial symbiont population dynamics (Chapter 2).

The null hypotheses for these two chapters are as listed below:

**Chapter 1:** Symbiont abundance in *Thyasira* cf. *gouldi* is constant throughout the year; there is no seasonal effect on symbiont population size.

**Chapter 2:** Bacterial symbionts do not undergo division in association with host gill epithelial cells; hosts can control bacterial population dynamics, and addition of reduced sulphur compounds does not result in an increase in bacterial division or population size.

## Co-Authorship Statement

Dr. Suzanne Dufour has been granted co-author status in each of two research chapters. In both cases, she contributed greatly to the design and identification of the research project, and assisted me in the acquisition of skills required for the practical application of the research. Data analysis and manuscript preparation were performed exclusively by myself, albeit with significant input from Dr. Dufour.

Additionally, Dr. Christine Paillard has been granted co-author status for Chapter 2, on the subject of bacterial symbiont division and population regulation by host thyasirids. The original identification of this question as one of interest took place in the summer of 2013 while I was working in her laboratory at the Institut Universitaire Européen de la Mer in Brest, France. Though the design of the experiment was conceived by me, she assisted in the refining of the eventual experimental design and provided assistance in the conducting of my first experimental trial on *Thyasira flexuosa*.

Finally, two papers have been included as appendices to my Master's thesis. Both of these are recent publications coming out of our lab to which I have made significant contributions. In the Batstone et al (2014) paper, I was responsible for producing the morphological data (light and transmission electron microscopy) included in the manuscript. It was this morphological data that originally indicated the presence of a cryptic species complex of *Thyasira* cf. *gouldi* in Bonne Bay, NL. As such, I was instrumental in identifying the research questions included in this paper, as well as in carrying out the practical aspect of morphological investigations and the analysis of morphological characteristics of host gill filaments and bacterial symbionts. Thus, I have

been included in this paper as second author, and though not an official chapter of my Master's thesis, the full paper is included in the appendix section (Appendix 1).

The Dufour et al (2014) paper, concerning the discovery of magnetosomes in the symbionts of *Thyasira* cf. *gouldi*, also included major contributions from my research. Magnetosomes were first putatively identified through my work on transmission electron microscopy pertaining to my other research projects, and I was instrumental in identifying and pursuing this research question. Furthermore, I contributed to the paper through transmission electron microscopy data regarding magnetosome morphology, as well as assistance in the investigation of magnetosomes through atomic force microscopy. As such, I have been included in this paper as second author, and though not an official chapter of my Master's thesis, the full paper is included in the appendix section (Appendix 2).

# **Chapter 1: Seasonal variation in symbiont abundance in the thyasirid bivalve *Thyasira cf. gouldi***

## **Authors:**

Laurich, J.R., Department of Biology, Memorial University of Newfoundland, St. John's, NL, Canada, A1B 3X9

Dufour, S.C., Department of Biology, Memorial University of Newfoundland, St. John's, NL, Canada, A1B 3X9

## **Abstract**

Within the bivalve family Thyasiridae, symbioses with chemoautotrophic, sulphur-oxidizing bacteria do not occur in all lineages; variation in symbiont presence and in the degree of abfrontal expansion of gill filaments occur on fine phylogenetic scales within the family. Thyasirid symbionts are housed extracellularly and are periodically engulfed and digested by host gill epithelial cells. Symbiotic thyasirids are mixotrophic, retaining the capacity to feed on particulate matter; the relative importance of particulate feeding and symbiont-derived nutrition to host metabolism may vary on temporal and spatial scales depending on the abundance of particulate organic matter and sediment sulphide availability. Here, we demonstrate the existence of a strong cyclical trend in symbiont abundance in *Thyasira cf. gouldi* from Bonne Bay, Newfoundland: symbiont abundance is highest during the months of autumn, and is lowest in late winter and spring. The density of membrane whorls, lysosomal microbodies associated with the

digestion of bacterial symbionts, may display a similar temporal trend, indicating that the relative contribution of symbiont-derived nutrition is likely contingent on seasonal fluctuations in environmental parameters. Symbiont abundance is correlated with shell size, and environmental cycles and/or shifts in the supply of sediment sulphides and particulate organic matter may influence thyasirid nutritional strategies and fitness. Along with the extracellular status of thyasirid symbioses, their highly dynamic nature may explain the evolutionary loss of chemosymbioses in this group.

## **Introduction**

Symbioses between chemoautotrophic bacteria and marine invertebrates are widespread and show extensive variation in the degree of host reliance on symbiont-derived nutrition (Dubilier et al. 2008, Chaston & Goodrich-Blair 2010). One of the invertebrate taxa in which chemosymbiosis varies greatly is the Bivalvia (Taylor & Glover 2006, Duperron et al. 2012, Roeselers & Newton 2012). Bivalve chemosymbioses run the gamut from the vertically transmitted, obligate associations where symbiont metabolism provides nearly all of the hosts' nutrition, as described in solemyids (Krueger et al. 1996, Stewart & Cavanaugh 2006), to the environmentally transmitted and nutritionally flexible symbioses seen in lucinids (Taylor & Glover 2006, Caro et al. 2009).

The Thyasiridae is one of two bivalve families known to have extracellular chemoautotrophic symbionts (Duperron et al. 2012), with bacteria housed in enlarged pockets defined by extensions of the host gill epithelial cell membrane (Dufour 2005).

The extracellular localization of symbionts is in stark contrast with other chemosymbiotic bivalve families in which bacteria are housed intracellularly (Taylor & Glover 2006, Roeselers & Newton 2012). Furthermore, not all members of the Thyasiridae possess symbionts, and this variation can occur on very fine phylogenetic scales: the genus *Thyasira* contains both species with and without symbionts (Dando & Southward 1986, Southward 1986), as does the cryptic *T. cf. gouldi* species complex (Batstone et al. 2014). Symbiosis in thyasirids is associated with extensive variation in host morphology, notably the abfrontal expansion of gill filaments and digestive tract reduction (Reid & Brand 1986, Dufour 2005). Marked differences in symbiont presence in thyasirids, coupled with the extracellular localization of symbionts and evolutionarily distinct symbiont acquisition events (Rodrigues & Duperron 2011) have led authors to postulate that thyasirid bivalves represent early stages of the evolution of complex chemosymbioses (Roeselers & Newton 2012).

Among symbiotic thyasirids, there is considerable variation in the importance of nutritional input from their symbionts. Unlike in other obligate associations where host invertebrates lack mouths, guts, and the capacity to feed (Stewart & Cavanaugh 2006, Nyholm et al. 2012), symbiotic thyasirids are capable of processing particulate food with their (reduced) palps and simplified digestive tracts (Allen 1958). In *Thyasira sarsi*, changes in environmental parameters associated with organic matter input and sediment sulphide concentrations can result in pronounced shifts in host nutritional strategy, ranging from almost complete dependence on symbiotically-derived nutrition to an essentially exclusive reliance on particulate organic matter (Dando & Spiro 1993). Additionally, the relative importance of symbiont-derived nutrition may vary greatly in *T.*

*flexuosa* as a function of seasonal cycles (Donval et al. 1989), and experimental manipulation of thyasirids has revealed that symbiont abundance is related to the availability of particulate food and sulphides (Dufour & Felbeck 2006).

In symbiotic thyasirids, transfer of nutrients from bacterial symbionts to host cells is thought to occur via symbiont endocytosis and lysosomal degradation (Southward 1986, Le Pennec et al. 1988, Dufour et al. 2014). Increased digestion of bacteria in starved symbiotic bivalves has been demonstrated in thyasirids (Dufour & Felbeck 2006), as well as in lucinids (Caro et al. 2009) and bathymodiolin mussels (Kádár et al. 2008). Symbiont digestion in these organisms results in the accumulation of lysosomal microbodies in the basal portion of host bacteriocyte cytoplasm. These structures, called “membrane whorls”, consist of the densely-packed remains of digested bacterial symbionts (Frenkiel et al. 1996, Liberge et al. 2001, Kádár et al. 2008).

Though there is evidence for temporal variability in the relative nutritional importance of symbiont metabolism to thyasirids (Donval et al. 1989, Dando & Spiro 1993, Dufour & Felbeck 2006), a systematic investigation of possible seasonal trends in symbiont abundance and the extent of bacterial digestion in natural populations of thyasirid bivalves has yet to be conducted. Here, we compare the symbiont abundance and density of membrane whorls in *Thyasira* cf. *gouldi* from Bonne Bay, Newfoundland, Canada, sampled at various times over two years. This subarctic fjord experiences seasonal fluctuations in photosynthetically-derived carbon (Tian et al. 2001) that could influence the availability of particulate organic matter and sulphides to *T. cf. gouldi*. Such temporal heterogeneity may be sufficient to produce variation in symbiont abundance in these thyasirids.



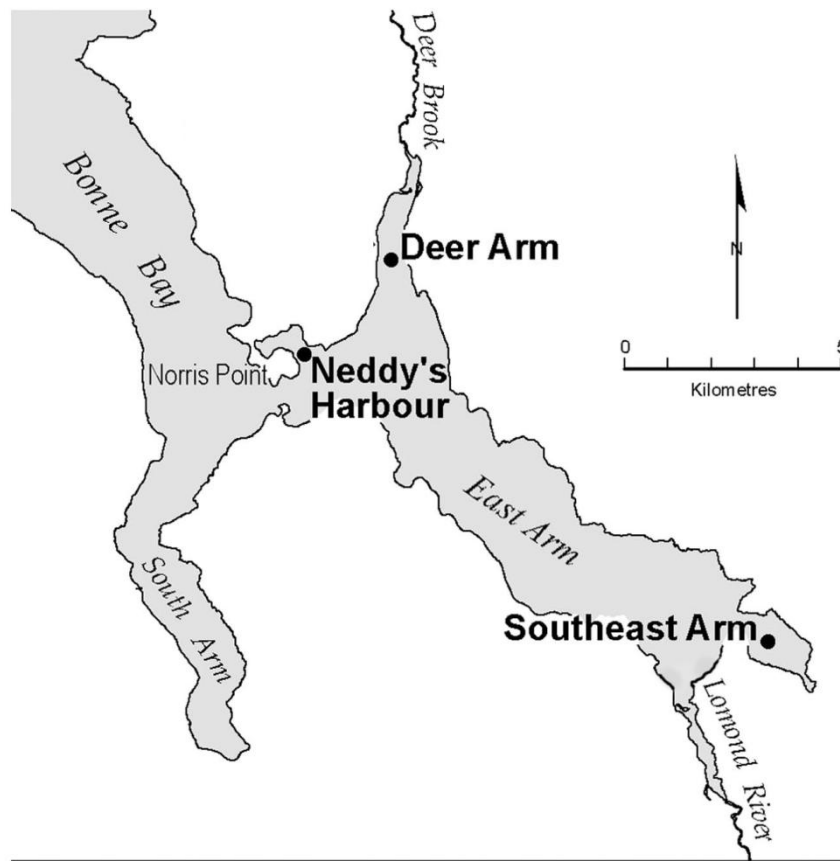
## Materials and Methods

### Sample collection

*Thyasira* cf. *gouldi* were collected in Bonne Bay, a subarctic fjord on the western coast of Newfoundland, Canada. Sediment and bivalves were collected from the sheltered, eastern basin of this fjord, a relatively isolated and stable body of water due to the presence of a shallow sill separating it from the Gulf of St. Lawrence (Gilbert & Pettigrew 1993). Samples were obtained from three sites: South East Arm (N 49°27.75, W 57°42.82), Deer Arm (N 49°33.21, W 57°50.42) and Neddy's Harbour (N 49°52.38, W 57°52.27, Fig 1). Collection sites are characterized by elevated levels of organic matter due to terrestrial plant debris via freshwater runoff (South East Arm and Deer Arm) and anthropogenic input from surrounding communities (Neddy's Harbour).

Thyasirids were collected over the course of 8 sampling trips in 2011 and 2012. Sediment was collected using a Peterson grab, and thyasirids were retrieved on a sieve with a 1 mm mesh. The anterior-posterior shell length of all thyasirids was measured prior to dissection and gill fixation.

The selection of *Thyasira* cf. *gouldi* to be used in this study was complicated by the existence of a cryptic species complex in Bonne Bay. Symbiotic *T. cf. gouldi* (i.e., OTUs 1 and 2) were identified using external morphological characteristics, including dorso-ventral elongation of the shell and the dorsal location of iron deposits on the shells of symbiotic *T. cf. gouldi* (Batstone et al. 2014). Only specimens confirmed to be symbiotic based on gill structure and transmission electron microscopy were used for quantitative analyses.



**Figure 1.** Map showing the location of the three sampling locations in Bonne Bay, Newfoundland. River input and the local community (Norris Point) are shown. Adapted from Batstone *et al.*, 2014.

Due to the somewhat opportunistic nature and of the sampling and patchy distribution of symbiotic *T. cf. gouldi* within the sediments of the three sampling sites, the number of individuals collected varied greatly between sites and sampling periods. In total, 48 clams were collected in 2011 and 56 in 2012, for a total sample size of 104 symbiotic *T. cf. gouldi* (Table 1).

**Table 1.** Number of symbiotic *Thyasira* cf. *gouldi* specimens used for quantitative and statistical analysis over the course of the 2011 and 2012 sampling seasons, from each of the three sampling sites.

Collection Date	Deer Arm	Neddy's Harbour	South East Arm
Apr 19, 2011	0	1	5
Jun 6, 2011	2	5	3
Aug 16, 2011	1	5	3
Oct 4, 2011	2	1	5
Dec 5, 2011	2	0	13
May 8, 2012	5	4	9
Jul 30, 2012	4	6	5
Oct 9, 2012	8	14	1

### **Gill fixation and transmission electron microscopy**

Gills were fixed in 2.5% gluteraldehyde in a 0.1 M sodium cacodylate buffer for 4 h, post-fixed with 1% osmium tetroxide for 1 h, and dehydrated in an increasing ethanol series prior to embedding in EPON resin. Ultra-thin sections (~60-70 nm) were cut on an ultramicrotome and placed on copper grids. Sections were then post-stained with uranyl acetate and lead citrate and examined on a Philips 300 transmission electron microscope.

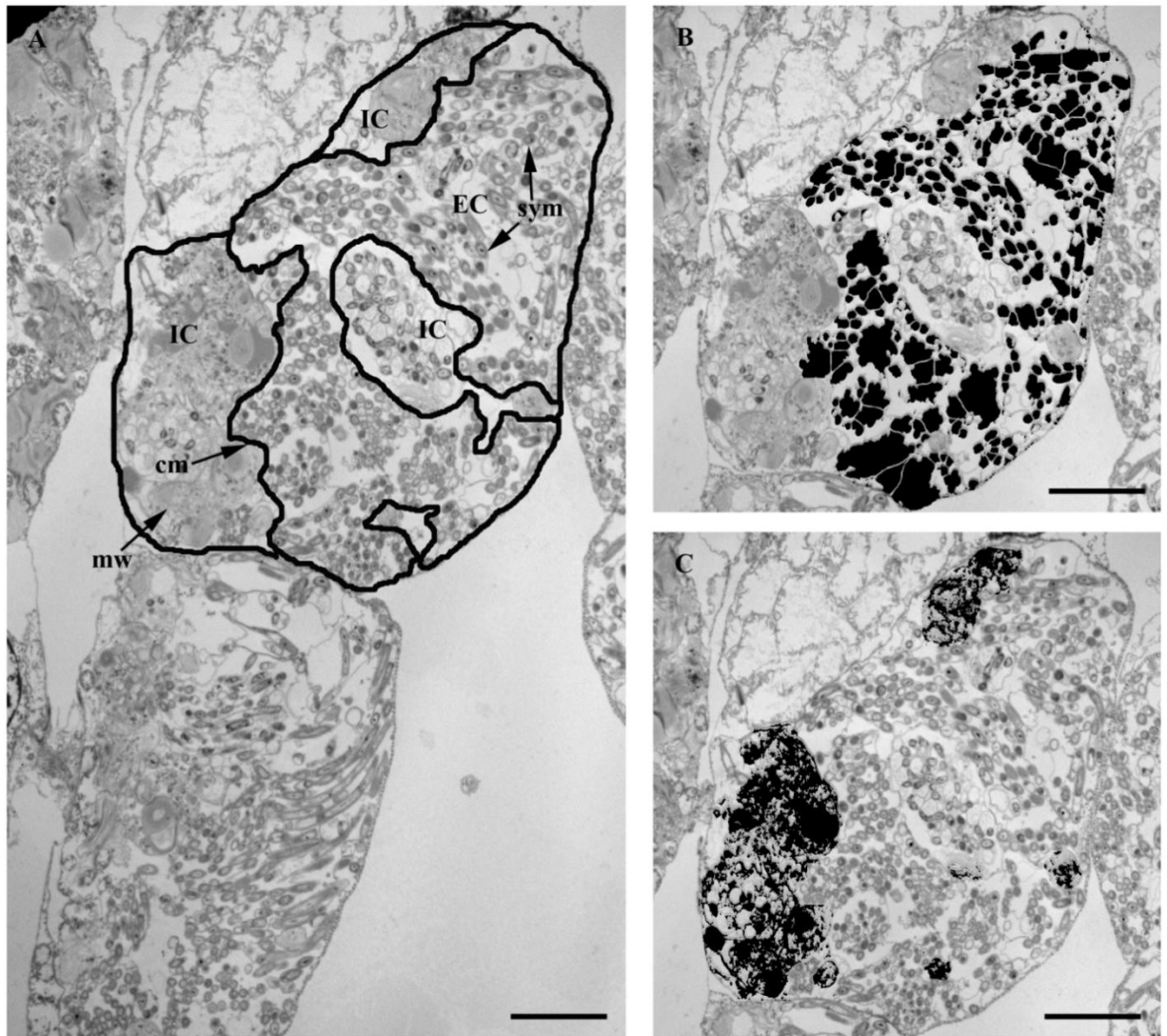
### **Quantitative analysis**

Transmission electron micrographs were taken of the frontal-most two bacteriocytes of two gill filaments per individual, for a total of four bacteriocytes per specimen. The two sets of bacteriocytes per specimen were chosen from different gill filaments to prevent potential bias associated with the analysis of four bacteriocytes from a single filament. Images were taken such that the entirety of the cell, including both intracellular and the extracellular, symbiont-containing portions could be visualized (Fig 2A).

Measurements used to establish the relative spatial occupation of symbionts and membrane whorls were made in ImageJ (Rasband 1997); the two-dimensional space occupied by symbionts and membrane whorls relative to the intracellular area of a bacteriocyte (considered to be relatively constant over time) were used as proxies for their abundance. The total cell area ( $T_A$ ), encompassing both intra- and extracellular regions was measured after manually outlining the area enclosed by the host cell membrane and the microvillar band that encloses the symbionts (Dufour 2005). Subsequently, the extracellular, symbiont-housing area ( $EC_A$ ) of the bacteriocytes was measured and cropped, and the intracellular area ( $IC_A$ ) was determined by subtracting this value from the total cell area ( $IC_A = T_A - EC_A$ ). The extracellular portion of the image was thresholded to leave no pixelated values except those associated with bacterial cell envelopes and cellular inclusions. Holes were filled such that the entirety of space occupied by individual bacterial cells was rendered to an above-threshold level (Fig 2B). Though this did result in an over-representation of the area occupied by bacterial symbionts due to the filling of holes between symbionts and other cellular structures such as the cell membrane, this error was encountered on all images examined in an approximately equivalent fashion. Thus, it did not interfere with the statistical analysis of variation in the spatial occupation of bacterial cells between different individuals. Subsequently, symbiont abundance ( $S_{Abd}$ ) was calculated as a function of the area occupied by bacterial symbiont cells ( $B_A$ ) divided by the total intracellular area of the host cell ( $S_{Abd} = B_A / IC_A$ ). Membrane whorl density ( $MW_D$ ) was calculated as a function of the spatial extent of membrane whorl occupation ( $MW_A$ ) and intracellular area ( $MW_D = MW_A / IC_A$ ), following the use of a similar thresholding on the intracellular portion of

host gill cells. Non-membrane whorl cellular features such as nuclei, lipid deposits and mitochondria were manually deleted prior to the measurement of membrane whorl area (Fig 2C). Both  $S_{Abd}$  and  $MW_D$  values are unitless, as they are a function of spatial occupation ( $cm^2$ ) per unit of host cell intracellular area (i.e.,  $cm^2/cm^2$ ).

Prior to statistical analysis, the values obtained for the 4 bacteriocytes of each individual were averaged to prevent an inflation of sample size and violation of the assumption of independence of variance.



**Figure 2.** Transmission electron micrographs of a *Thyasira cf. gouldi* gill filament. All scale bars represent 5  $\mu\text{m}$ . (A) Frontal-most two bacteriocytes of a gill filament. The cell membrane (cm) of the frontal-most cell is traced in black to illustrate the location of the intracellular (IC) and extracellular (EC) zones. Symbiotic bacteria (sym) and membrane whorls (mw) are seen. (B-C) Micrographs of the bacteriocyte with traced cell membrane in (A). (B) The symbiotic bacteria have been rendered binary and had their holes filled to calculate occupation area. The results of this process can be seen in black. (C) The intracellular area has been thresholded so as to measure membrane whorl spatial occupation. The results of this process can be seen in black.

## Statistical analyses

Potential effects of sampling date, sampling site and host shell length on  $S_{Abd}$  and  $MW_D$  were examined using ANCOVAs and linear regression models. For these analyses, sampling date and site were treated as categorical variables. Statistical analyses were carried out in R (version 3.10, R Development Core Team 2014). The results of type III ANOVAs and ANCOVAs involving multiple explanatory variables were produced using the “car” package (Fox & Weisberg 2011).

The effect of sampling site on  $S_{Abd}$  and  $MW_D$  was confounded by the presence of smaller individuals at Neddy’s Harbour (average shell length  $\pm$  standard error:  $2.87 \pm 0.20$  mm) than at South East Arm (average shell length  $\pm$  standard error:  $3.77 \pm 0.24$  mm) and Deer Arm (average shell length  $\pm$  standard error:  $3.78 \pm 0.27$  mm). Given that the difference in shell length between sites was statistically significant (ANOVA,  $p < 0.001$ ), and that shell length correlates with  $S_{Abd}$  (see below), the size-corrected effect of sampling site on  $S_{Abd}$  and  $MW_D$  became highly insignificant (ANCOVA,  $p = 0.67$ ,  $p = 0.99$ , respectively). As such, the final linear models constructed for  $S_{Abd}$  and  $MW_D$  in *Thyasira* cf. *gouldi* considered sampling date and shell length only (not sampling site). The relationship between  $MW_D$  and  $S_{Abd}$  was also assessed using linear regression models.

Apparent differences in  $S_{Abd}$  between 2011 and 2012 were evaluated using a linear model incorporating the year (2011 or 2012) as well as the numeric value for day of the year corresponding to each sampling date. After finding significant inter-annual variation in  $S_{Abd}$  (ANCOVA,  $p < 0.01$ ) after accounting for the continuous effect of the numeric value for the day of the year, a model describing variation in  $MW_D$  as a function of

sampling date was constructed using data from 2011, the year in which more variation was uncovered.

### **Generalized additive model construction**

To better reflect the cyclical influence of time on  $S_{Abd}$ , generalized additive models (GAMs) were constructed using the “mgcv” package in R (Wood 2011). Three GAMs were constructed to evaluate the role of shell length, sampling site and date on  $S_{Abd}$  in *Thyasira cf. gouldi* (models 1-3, Table 2). The use of smoothing functions on explanatory variables is indicated by “s( )”. “Day” represents the effective date in days since December 31<sup>st</sup> 2010 and is treated as a continuous variable, while “Length” and “Site” refer to shell length and collection site, respectively. Interactions between terms are indicated by “\*”. The number of knots used in GAM construction was set at 4, to fit a simplistic model that covers one seasonal minima and maxima in symbiont abundance as well as two endpoints. The three GAMs were compared to the linear model of variation in  $S_{Abd}$  as a function of sampling date (“Month”, treated as a fixed factor) and shell length (model 4, Table 2).

Model selection was based on the use of the Akaike Information Criterion (AIC). Following selection of the best model by identifying the model with the lowest AIC score (Burnham & Anderson, 2002), an artificial dataset was produced for the 539 days between the first and final sampling trips. The average of thyasirid shell length was calculated across all examined specimen, and this value was incorporated into the best model (model 2, Table 2, AIC score = 90.994) in order to predict minimal and maximal  $S_{Abd}$  values and to evaluate the degree of variation in  $S_{Abd}$  predicted by the model. Finally,



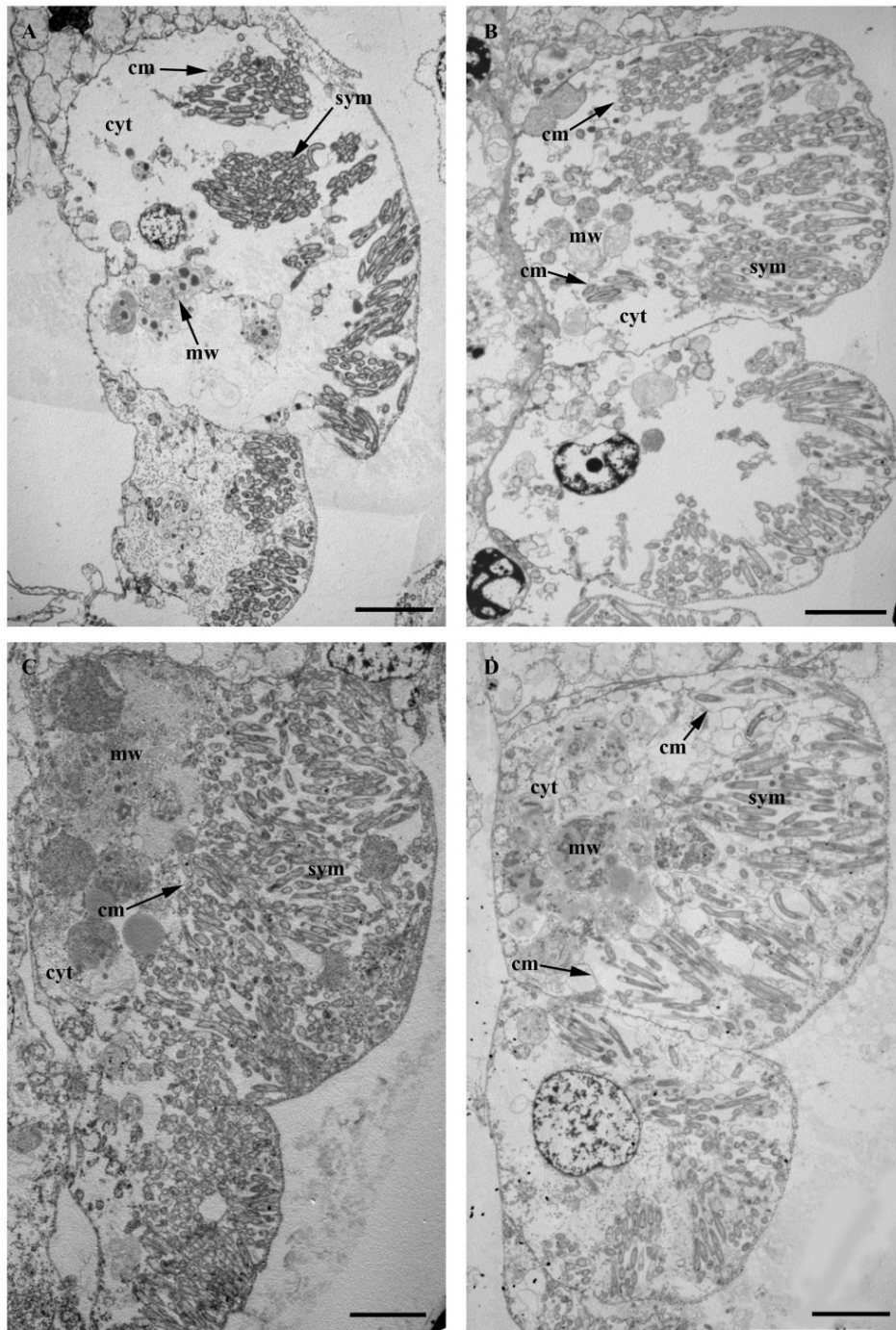
a GAM was constructed for variation in  $MW_D$ , using model parameters based on the best  $S_{Abd}$  GAM, as determined by the use of AIC.

## Results

*Thyasira cf. gouldi* gill structure and cellular characteristics were consistent with the type 3 gill organization of Dufour (2005), as previously described in Batstone et al. (2014). Symbiotic bacteria were housed extracellularly in pockets delineated by host microvillar bands and evaginations of the cell membrane and cytoplasm (Fig 2A). Symbionts were associated with the epithelial cells of abfrontally expanded gill filaments, in all specimens with type 3 gills examined.

### Temporal variability in symbiont abundance

Symbiont abundance in the extracellular space of *Thyasira cf. gouldi* bacteriocytes displayed a remarkable amount of temporal variation. In individuals collected in April 2011, symbionts were largely restricted to relatively small extracellular pockets (Fig 3A). Relative bacterial abundance increased over the course of early summer, and by June 2011, symbionts were noticeably more abundant, occupying larger extracellular pockets (Fig 3B). Symbiont density was exceptionally high in August 2011 and remained at similar levels through October and December (Fig 3C). Sampling in May 2012 revealed a return to less abundant symbionts in *T. cf. gouldi* bacteriocytes (Fig 3D). Membrane whorls were present in individuals collected at all dates, though there was an apparent increase in the relative density of these structures in the cytoplasm of host cells over the course of the 2011 sampling season (Fig 3).

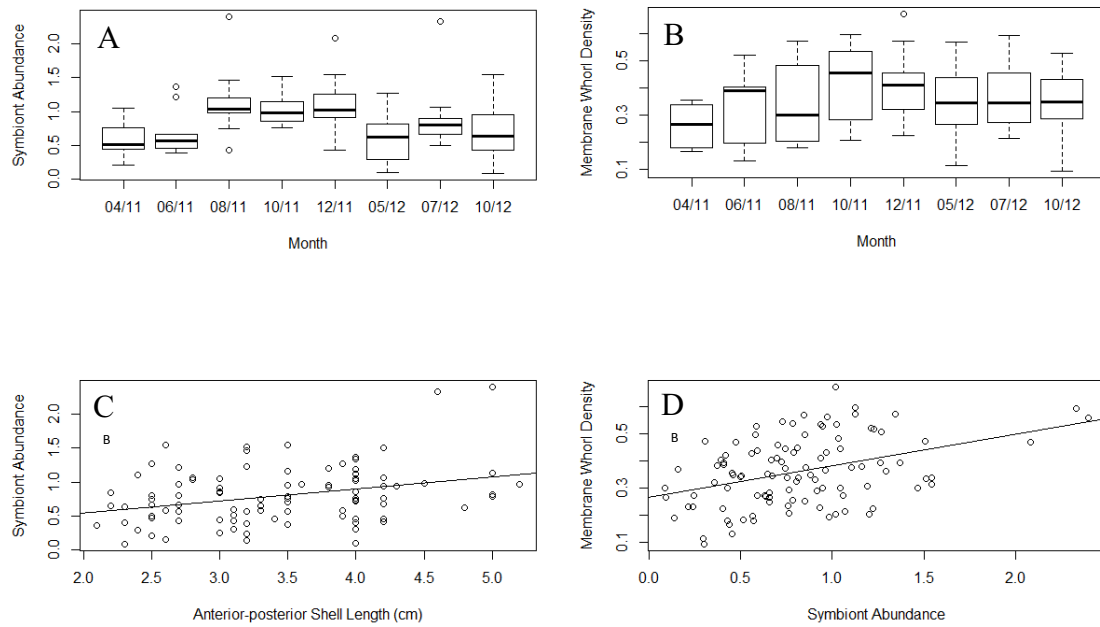


**Figure 3.** Transmission electron micrographs of *Thyasira cf. gouldi* gill filaments. Symbionts (sym) can be seen in the extracellular pockets formed by extensions of the host cell membrane (cm). Membrane whorls (mw) can be seen within the host cytoplasm (cyt). All scale bars represent 5 μm. (A) Micrograph of specimen collected in April 2011. (B) Micrograph of specimen collected in June 2011. (C) Micrograph of specimen collected in December 2011. (D) Micrograph of specimen collected in May 2012.

ANCOVA revealed significant effects of sampling date on  $S_{Abd}$  (Fig 4A;  $p < 0.01$ ), but not on  $MW_D$  (Fig 4B;  $p = 0.23$ ). Significant differences in  $S_{Abd}$  were found between 2011 and 2012 after accounting for the effect of day of the year ( $p < 0.01$ ), indicating inter-annual variation in *Thyasira* cf. *gouldi* symbiont abundance in Bonne Bay. However, patterns in a given year were similar: in 2011,  $S_{Abd}$  was lowest in April ( $0.58 \pm 0.11$ ) and highest in early August ( $1.16 \pm 0.21$ ), and in 2012, it was lowest in May ( $0.59 \pm 0.13$ ) and highest in late July ( $0.87 \pm 0.16$ ). Minimal and maximal average  $MW_D$  coincided temporally with minima and maxima in  $S_{Abd}$ . In 2011,  $MW_D$  ranged from  $0.26 \pm 0.03$  in April to  $0.42 \pm 0.06$  in October. However, variation in  $MW_D$  was much less pronounced in 2012, with a minimum of  $0.35 \pm 0.05$  in May and a maximum of  $0.38 \pm 0.04$  in July.

### **Factors influencing symbiont abundance and membrane whorl density**

Symbiont abundance varied linearly as a function of shell length (Fig 4C,  $p < 0.01$ ), increasing at a rate of  $0.18 \pm 0.05 \text{ mm}^{-1}$ , with a Pearson correlation coefficient of 0.323. Membrane whorl density correlated with shell length ( $p < 0.01$ ) and with  $S_{Abd}$  (Fig 4D,  $p < 0.001$ ,  $R = 0.398$ ). Interestingly, there was no statistically significant effect of sampling date on  $MW_D$  ( $p = 0.23$ ), despite the strong effect of sampling date on  $S_{Abd}$  ( $p < 0.01$ ). It is worth noting that the slope of the correlation between  $S_{Abd}$  and  $MW_D$  was quite low ( $0.12 \pm 0.03$ ), raising the possibility that a seasonal trend in  $MW_D$  (corresponding to that observed for  $S_{Abd}$ ) does exist but is too slight to detect.



**Figure 4.** (A) Variation in symbiont abundance over the 8 sample collection dates. Month has an effect on bacterial symbiont abundance (ANCOVA,  $p = 0.006372$ ). (B) Variation in membrane whorl density over the course of sample collection. Month has no significant effect on membrane whorl density (ANCOVA,  $p = 0.22698$ ). (C) A significant correlation ( $p = 0.00118$ ,  $R = 0.322977$ ) exists between symbiont abundance and anterior-posterior shell length. (D) There is a significant ( $p = 0.0000284$ ,  $R = 0.3981945$ ) correlation between membrane whorl density and bacterial symbiont abundance.

### Symbiont abundance and membrane whorl density: generalized additive models

Three GAMs, along with a fourth linear model, were compared to explore variation in  $S_{Abd}$  as a function of time (Table 2). All four models provided considerable evidence for an effect of time on  $S_{Abd}$  ( $p < 0.01$ ). Based on the AIC scores, model 2 was the best at explaining the variation in  $S_{Abd}$  (Fig 5). Model 1 was discounted, as its  $\Delta AIC$  (the difference between its AIC score and the AIC score of the best model) of 3.781 was  $> 2$ , an indication that little evidence exists to substantiate it (Burnham & Anderson 2002). In contrast, neither model 3 nor 4 could be rejected, as their  $\Delta AIC$  of 1.724 and

1.755 respectively were both  $< 2$  (Burnham & Anderson 2002). The model best supported by our data was based on a cyclical function of sampling date, and both models 2 and 3 were better supported than the linear model treating time as a fixed factor (Table 2).

Therefore, variation in  $S_{Abd}$  in *Thyasira* cf. *gouldi* is a function of annually occurring temporal cycles, with an additional, significant effect of shell length ( $p < 0.01$ , model 2).

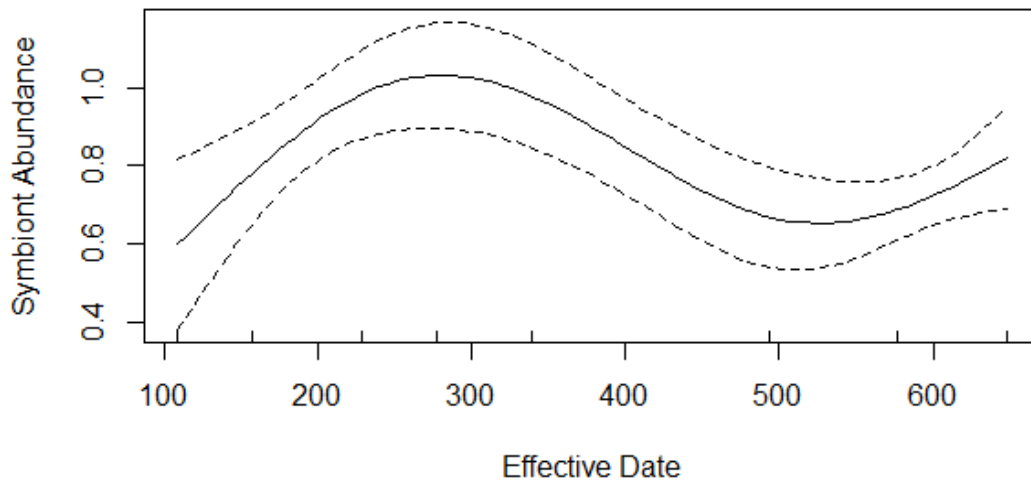
An interactive term between shell length and date may also help to explain the variation in  $S_{Abd}$  (model 3).

**Table 2.** Model selection for the influence of time on bacterial abundance. 3 generalized additive models (1-3), with smoothing functions “s()” applied to the continuous effect of time, and one linear model (4) were considered. The incorporation of interactive effects between terms is indicated by a “\*”. Relevant p-values and Akaike Information Criterion (AIC) scores are presented.

Model Formula	p-value (Effect of Day)	AIC Score
(1) $S_{Abd} \sim s(\text{Day}) + \text{Size} + \text{Site}$	0.00541	94.775
(2) $S_{Abd} \sim s(\text{Day}) + \text{Size}$	0.00518	90.994
(3) $S_{Abd} \sim s(\text{Day}) + \text{Size} + \text{Size} * \text{Day}$	0.00718	92.718
(4) $S_{Abd} \sim \text{Month} + \text{Size}$	0.000223	92.749

As model 2 was determined to be the best, it was used to make predictions relating to variation of symbiont abundance of the Bonne Bay *Thyasira* cf. *gouldi* population (Fig 5). Based on the portion of the model over which continuous data collection coincided with the predicted trend in  $S_{Abd}$ , a seasonal peak can be predicted for early October (Oct 8, 2011,  $S_{Abd} = 1.031$ ), while symbiont abundance is predicted to be at its lowest in late February (Feb 22, 2012,  $S_{Abd} = 0.6523$ ). These values predict a 58.06% increase in relative symbiont abundance between a late winter minimum and autumn maximum in the bacteriocytes of *T. cf. gouldi* in Bonne Bay.

Investigation of a potentially non-linear effect of day on  $MW_D$  was evaluated based on the best model 2 for variation in  $S_{Abd}$ . Use of non-linear modeling techniques failed to modify the outcomes of linear comparisons of  $MW_D$  at various sampling times, and there was no evidence for an effect of time on  $MW_D$  ( $p = 0.32$ ). However, exclusion of the 2012 sampling year data on  $MW_D$  (for which substantially less variation was observed, Fig 4B) in a linear model describing  $MW_D$  resulted in a significant effect of sampling date on  $MW_D$  ( $p < 0.05$ ). Therefore, a seasonal trend in  $MW_D$  may exist in *Thyasira cf. gouldi*, but its significance is obscured by inter-annual variation and a relative lack of variation in  $MW_D$  over the course of 2012.



**Figure 5.** Generalized additive model for variation in symbiont abundance in *Thyasira cf. gouldi* as a cyclical function of time. The model presented was selected based on Akaike Information Criterion (Table 2). Collection date, represented as effective date (days since Dec 2010), has a significant effect on symbiont abundance ( $p = 0.00518$ ). Additionally, the effect of shell size was significant ( $p = 0.00222$ ).

## Discussion

*Thyasira* cf. *gouldi* exhibits a remarkable degree of seasonal variation in the abundance of its sulphur-oxidizing bacterial symbionts. This variation is readily observed on transmission electron micrographs and was validated through the implementation of models, all of which revealed significant effects of sampling date on symbiont abundance ( $p < 0.01$ ). Variation in symbiont abundance was best represented as a non-linear effect of time, with a cycle corresponding to a yearly low in late winter and a maximum in autumn. As *T. gouldi* is considered to be a pan-arctic species (Oliver et al. 2002, Włodarska-Kowalczyk 2007) inhabiting regions with seasonal fluctuations similar to those in Bonne Bay, seasonal trends in symbiont abundance likely also exist in other populations. Furthermore, the congruence of the predicted seasonal maxima and minima with a seasonal cycle previously postulated for *T. flexuosa* (Donval et al. 1989) on the basis of variation in digestive gland morphology indicates that this trend may be relevant to many symbiotic thyasirid species.

A less pronounced degree of seasonal variation in membrane whorl density within the Bonne Bay *Thyasira* cf. *gouldi* populations was seen upon visual inspection of electron micrographs. A linear model that excluded the 2012 sampling year, in which there was significantly less variation in symbiont abundance than in 2011, revealed a significant effect ( $p < 0.05$ ) of time on the density membrane whorls, consistent with a significant correlation between  $MW_D$  and  $S_{Abd}$ . As membrane whorls are interpreted as evidence of nutrient transfer from symbiont to host through bacterial cell lysis (Southward 1986, Le Pennec et al. 1988, Kádár et al. 2008), potential seasonal variation

in MW<sub>D</sub> should indicate fluctuations in the nutritional input derived from symbiotic metabolism, as documented in Swedish *T. sarsi* populations (Dando & Spiro 1993).  $\delta^{13}\text{C}$  ratios in *T. sarsi* revealed striking differences in the importance of symbiotically derived nutrition associated with variation in sediment sulphide in a cyclically anoxic, anthropogenically-polluted basin.

In Bonne Bay, there are seasonal cycles in the vertical export of photosynthetically derived nutrients, with a greater organic matter input to the benthos in early spring and a slight spike associated with a second phytoplankton bloom in late summer (Tian et al. 2001). Accumulation of organic matter in sediments can lead to an increase in the activity and abundance of sulphate-reducing bacteria (Goldhaber & Kaplan 1975, Westrich & Berner 1984) and the accumulation of sedimentary sulphides (Mudryk et al. 2000). Seasonal fluctuations in the abundance of sedimentary sulphate-reducing bacteria were observed in the Baltic Sea (Mudryk et al. 2000) and in simulated wetland microcosms (Faulwetter et al. 2013), with highest and lowest rates of sulphate reduction occurring in late summer and spring, respectively. The concentration of sediment sulphides has been shown to affect thiasirid symbiont abundance (Dufour & Felbeck 2006), and could thus explain the temporal cycle observed in the present study. In Bonne Bay sediments, reduced sulphate reduction rates in the spring (and relatively lower sedimentary sulphide reserves) could restrict the capacity of thiasirids to harbour large symbiont populations during those months. A subsequent increase in sulphate reduction rates over the course of summer would increase sedimentary sulphide concentrations, and coincides with an observed increase in *T. cf. gouldi* symbiont populations.



Declining symbiont abundance in late winter could also be a consequence of sediment sulphide depletion due to the extensive sulphide mining behaviour of symbiotic thyasirids (Dufour & Felbeck 2003). Symbiotic thyasirids are considered to be ecological engineers as their burrow construction and ventilation activities result in enhanced oxygenation and sulphide depletion in surrounding sediments (Dando & Southward 1986, Dando & Spiro 1993, Dando et al. 2004). Similar effects of chemosymbiotic bivalves on sediment chemistry have been demonstrated for lucinids in *Thalassia testudinum* meadows, where 16% of sediment sulphide production is removed through lucinid sulphide consumption and through burrowing behaviours that re-introduce oxygen into sediments (Reynolds et al. 2007). In fact, lucinids are crucial in limiting sediment sulphide accumulation in seagrass beds, maintaining the basis of a three-stage symbiosis between them, the seagrasses that dominate their habitats, and their bacterial symbionts (van der Heide et al. 2012). In Bonne Bay, ice cover limits the input of particulate organic matter during winter months; a potentially greater nutritional reliance of the often dense *Thyasira cf. gouldi* populations upon their symbionts during that time could result in increased ventilation and sulphide mining behaviours (Dando & Southward 1986, Dando et al. 2004, Dufour & Felbeck 2006). Such behaviours could lead to the depletion of sediment sulphides prior to the disappearance of ice cover and the subsequent spring phytoplankton bloom.

Regardless of its cause, the *Thyasira cf. gouldi* population of Bonne Bay undergoes significant variation in symbiont density, with a predicted 58.06% increase in symbiont abundance between late winter and autumn. Therefore, seasonally fluctuating characteristics (most likely concentrations of reduced sulphur in sediments and/or

particulate organic matter) are influencing thyasirid symbiont abundance under natural conditions. This finding is consistent with previous experimental evidence for an effect of sediment sulphide and particulate organic matter on symbiont abundance in mixotrophic thyasirids (Dufour & Felbeck 2006). That *T. cf. gouldi* likely alters its nutritional strategy based on environmental parameters can help to explain the recent evolutionary loss of symbionts in some OTUs within the cryptic *T. cf. gouldi* species complex (Batstone et al. 2014). Short-term environmental changes, such as increased sewage input (Dando & Spiro 1993), could result in new evolutionary pressures on symbiotic thyasirids in a given habitat. Such pressures could lead to the evolution of more complex and obligate chemosymbioses if sulphide supply became heightened or constant, or to a reversion to an asymbiotic lifestyle if symbiosis becomes unsustainable (Batstone et al. 2014).

The observed correlation between *Thyasira cf. gouldi* symbiont abundance and shell length is consistent with the hypothesis that the input of symbiont-derived nutrition allows symbiotic thyasirids to attain larger sizes (Dufour 2005), with particulate feeding being supplemented by the digestion of bacterial symbionts. Alternatively, the correlation between shell size and bacterial abundance could reflect differences in the thyasirid life cycle, with juvenile clams harbouring lesser symbiont populations. Inclusion of an interaction term between shell length and sampling date in the construction of GAMs failed to produce a significant effect ( $p = 0.548$ ) but the possibility that seasonal cycles in thyasirid reproduction and growth affect symbiont abundance cannot be ruled out.

*Thyasira cf. gouldi* from Neddy's Harbour were significantly smaller, and housed noticeably smaller symbiont populations, than those from South East Arm and Deer Arm. The latter two sites are located at the mouths of rivers, and likely receive a greater input

of plant detritus from the surrounding environment and river systems than Neddy's Harbour, located in a relatively isolated bay surrounded by a local community. The relative lack of terrestrial organic matter input in Neddy's Harbour may be a size-limiting factor in this population. This stresses the importance of symbionts as nutritional supplements for thyasirids (Dufour 2005), which may derive significant fitness benefits by hosting larger symbiont populations.

## **Conclusions**

Symbiont abundance in the bacteriocytes of *T. cf. gouldi* exhibits a strong seasonal trend characterized by a peak in bacterial density in the months of late summer and autumn, followed by a decline beginning over the course of winter and culminating with a seasonal low during late winter and spring. This trend may be mirrored by variation in the density of membrane whorls, the lysosomal microbodies characterized by the remains of digested bacterial symbionts. This seasonal cycle, in which the input symbiotically-derived organic carbon to bivalve hosts is greater in the autumn is likely driven by temporal variation in the import of terrestrial and planktonic organic matter.

Bivalve size and reproductive fitness may be linked to symbiont abundance, providing a basis for strong selection pressures based on sediment sulphide concentrations and the input of organic matter. Long-term shifts in environmental parameters could potentially result in the loss of, or greater reliance upon, bacterial symbionts. The highly dynamic symbiosis documented in this study helps to explain the

phylogenetic patchiness of chemosymbiosis in the family Thyasiridae, as well as the potential for a rapid evolutionary reversion to an asymbiotic state.

## Works Cited

- Allen, J. (1958). On the basic form and adaptations to habitat in the Lucinacea (Eulamellibranchia). *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences*. 241:684, 421-484.
- Batstone, R.T., Laurich, J. R., Salvo, F., & Dufour, S. C. (2014). Divergent chemosymbiosis-related characters in *Thyasira* cf. *gouldi* (Bivalvia: Thyasiridae). *PLoS ONE*. 9:3, e92856.
- Burnham, K. P., & Anderson, D. R. (2002). Model selection and multimodel inference: a practical information-theoretic approach, 2nd edition. Springer-Verlag, New York.
- Caro, A., Got, P., Bouvy M., Troussellier, M., & Gros, O. (2009). Effects of long-term starvation on a host bivalve (*Codakia orbicularis*, Lucinidae) and its symbiont population. *Applied and Environmental Microbiology*. 75:10, 3304-3313.
- Chaston, J. & Goodrich-Blair, H. (2010). Common trends in mutualism revealed by model associations between invertebrates and bacteria. *FEMS Microbiology Reviews*. 34, 41-58.
- Dando, P. R., & Southward, A. J. (1986). Chemoautotrophy in bivalve molluscs of the genus *Thyasira*. *Journal of the Marine Biology Association of the United Kingdom*. 66: 915-929.
- Dando, P.R., Southward A. J., & Southward, E. C. (2004). Rates of sediment sulphide oxidation by the bivalve mollusc *Thyasira sarsi*. *Marine Ecology Progress Series*. 280, 181-187.
- Dando, P.R., & Spiro, B. (1993). Varying nutritional dependence of the thyasirid bivalves *Thyasira sarsi* and *T. equalis* on chemoautotrophic symbiotic bacteria, demonstrated by isotope ratios of tissue carbon and shell carbonate. *Marine Ecology Progress Series*. 92, 151-158.
- Donval, A., Le Pennec, M., Herry, A., & Diouris, M. (1989). Nutritional adaptations of littoral bivalve molluscs to reducing biotopes. In J. S. Ryland, & P. A. Tyler

(Eds.), *Reproduction, Genetics and Distributions of Marine Organisms. Proceedings of the 23rd European Marine Biology Symposium*. 373-378. Swansea.

- Dubilier, N, Bergin, C., & Lott, C. (2008). Symbiotic diversity in marine animals: the art of harnessing chemosynthesis. *Nature Reviews Microbiology*. 6:10, 725-740.
- Dufour, S. C. (2005). Gill anatomy and the evolution of symbiosis in the bivalve family Thyasiridae. *Biological Bulletin*. 208, 200-212.
- Dufour, S. C., & Felbeck, H. (2003). Sulphide mining by the superextensile foot of symbiotic thyasirid bivalves. *Nature*. 426:6962, 65-67.
- Dufour, S. C., & Felbeck, H. (2006). Symbiont abundance in thyasirids (Bivalvia) is related to particulate food and sulphide availability. *Marine Ecology Progress Series*. 320, 185-194.
- Dufour, S. C., Laurich, J. R., Batstone, R. T., McCuaig, B., Elliot, A., & Poduska, K. M. (2014). Magnetosome-containing bacteria living as symbionts of bivalves. *The ISME Journal*. doi:10.1038/ismej.2014.93.
- Duperron, S., Gaudron, S.M., Rodrigues C.F., Cunha M.R., Decker C, & Olu, K. (2012). An overview of chemosynthetic symbioses in bivalves from the North Atlantic and Mediterranean Sea. *Biogeosciences Discussions*. 9, 16815-16875.
- Faulwetter, J. L., Burr, M. D., Parker, A. E., Stein, O. R., & Camper, A. K. (2013). Influence of season and plant species on the abundance and diversity of sulfate reducing bacteria and ammonia oxidizing bacteria in constructed wetland microcosms. *Microbial Ecology*. 65:1, 11-127.
- Fox, J., & Weisberg, S. (2011). An {R} Companion to Applied Regression, Second Edition. Thousand Oaks CA: Sage. URL: <http://socserv.socsci.mcmaster.ca/jfox/Books/Companion>.
- Frenkiel, L., Gros, O., & Moueza, M. (1996). Gill structure in *Lucina pectinata* (Bivalvia: Lucinidae) with reference to hemoglobin in bivalves with symbiotic sulphur-oxidizing bacteria. *Marine Biology*. 125:3, 511-524.
- Gilbert, D., & Pettigrew, G. (1993). Current-meter data from Bonne Bay, Newfoundland during the summer of 1991. *Canadian Report of Hydrography and Ocean Sciences*. 122:63.
- Goldhaber, M. B., & Kaplan, I. R. (1975). Controls and consequences of sulfate reduction rates in recent marine sediments. *Soil Science*. 119, 42-55.

- Kádár, E., Davis, S. A., & Lobo-da-Cunha, A. (2008). Cytoenzymatic investigation of intracellular digestion in the symbiont-bearing hydrothermal bivalve *Bathymodiolus azoricus*. *Marine Biology*. 153, 995-1004.
- Krueger, D. M., Gustafson, R. G., & Cavanaugh, C. M. (1996). Vertical transmission of chemoautotrophic symbionts in the bivalve *Solemya velum* (Bivalvia: Protobranchia). *Biological Bulletin*. 190:2, 195-202.
- Le Pennec, M., Diouris, M., & Herry, A. (1988). Endocytosis and lysis of bacteria in gill epithelium of *Bathymodiolus thermophilus*, *Thyasira flexuosa*, and *Lucinella divaricata* (Bivalve, Molluscs). *Journal of Shellfish Research*. 7:3, 483-489.
- Liberge, M., Gros, O., & Frenkiel, L. (2001). Lysosomes and sulfide-oxidizing bodies in the bacteriocytes of *Lucina pectinata*, a cytochemical and microanalysis approach. *Marine Biology*. 139, 401-409.
- Mudryk, Z., Podgórska, B., Ameryk, A., & Bolalek, J. (2000). The occurrence and activity of sulphate - reducing bacteria in the bottom sediments of the Gulf of Gdańsk. *Oceanologia Acta*. 42, 105-117.
- Nyholm, S. V., Song, P., Dang, J., Bruce, C., & Girguis, P. R. (2012). Expression and putative function of innate immunity genes under *in situ* conditions in the symbiotic hydrothermal vent tubeworm *Ridgeia piscesae*. *PLoS ONE*. 7, e38267.
- Oliver, P. G., & Kileen, I. J. (2002). The Thyasiridae (Mollusca: Bivalvia) of the British continental shelf and North Sea oil fields: an identification manual. *Wales: Studies in Marine Biodiversity and Systematics from the National Museum of Wales. BIOMOR Reports*. 3, 1-73.
- Rasband, W. S. (1997). ImageJ, U.S. National Institutes of Health. Bethesda, Maryland, USA. <http://imagej.nih.gov/ij/>
- R Development Core Team. (2014). R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing. Vienna, Austria. <http://www.R-project.org>
- Reid, R. G. B., & Brand, D. G. (1986). Sulfide-oxidizing symbiosis in Lucinaceans: implications for bivalve evolution. *The Veliger*. 29:1, 3-24.
- Reynolds, L. K., Berg, P., & Zieman, J. C. (2007). Lucinid clam influence on the biogeochemistry of the seagrass *Thalassia testudinum* sediments. *Estuaries and Coasts*. 30:3, 482-490.

- Rodrigues, C. F., & Duperron, S. (2011). Distinct symbiont lineages in three thyasirid species (Bivalvia: Thyasiridae) from the eastern Atlantic and Mediterranean Sea. *Naturwissenschaften*. 98:4, 281-287.
- Roeselers, G., & Newton, I. L. G. (2012). On the evolutionary ecology of symbioses between chemosynthetic bacteria and bivalves. *Applied Microbiology and Biotechnology*. 94, 1-10.
- Southward, E. C. (1986). Gill symbionts in Thyasirids and other bivalve molluscs. *Journal of the Marine Biology Association of the United Kingdom*. 66, 889-914.
- Stewart, F.J., & Cavanaugh, C.M. (2006). Bacterial endosymbioses in *Solemya* (Mollusca: Bivalvia)-model systems for studies of symbiont-host adaptation. *Antonie van Leeuwenhoek*. 90, 343-360.
- Taylor, J. D., & Glover, E. A. (2006). Lucinidae (Bivalvia)- the most diverse group of chemosymbiotic molluscs. *Zoological Journal of the Linnaean Society*. 148, 421-438.
- Tian, R. C., Vézina, A. F., Starr, M., & Saucier, F. (2001). Seasonal dynamics of coastal ecosystems and export production at high latitudes: a modeling study. *Limnology and Oceanography*. 46:8, 1845-1859.
- van der Heide, T., Govers, L. L., de Fouw, J., Olff, H., van der Geest, M., van Katwijk, M. M., Piersma, T., van de Koppel, J., Silliman, B. R., Smolders, A. J. P., & van Gils, J. A. (2012). A three-stage symbiosis forms the foundation of seagrass ecosystems. *Science*. 336, 1432-1434.
- Westrich, J. T., & Berner, R. A. (1984). The role of sedimentary organic matter in bacterial sulfate reduction: the G model tested. *Limnology and Oceanography*. 29:2, 236-249.
- Włodarska-Kowalczyk, M. (2007). Molluscs in Kongsfjorden (Spitsbergen, Svalbard): A species list and patterns of distribution and diversity. *Polar Research*. 26, 48-63.
- Wood, S. N. (2011). Fast stable restricted maximum likelihood and marginal likelihood estimation of semiparametric generalized linear models. *Journal of the Royal Statistical Society. Series B (Statistical Methodology)*. 73:1, 3-36.

## **Chapter 2: Manipulating life and death in facultative chemosymbioses; control of bacterial population dynamics in the Thyasiridae**

### **Authors:**

Laurich, J.R., Department of Biology, Memorial University of Newfoundland, St. John's, NL, Canada, A1B 3X9

Dufour, S.C., Department of Biology, Memorial University of Newfoundland, St. John's, NL, Canada, A1B 3X9

Paillard, C., Laboratoire des Sciences de l'Environnement Marin, Institut Universitaire Européen de la Mer, Technopole Brest-Iroise, France, 29280 Plouzane.

### **Abstract**

Stable mutualistic associations between marine invertebrates and their chemosynthetic bacterial symbionts are predicated on both the adequate transfer of resources and on the restriction of bacterial cells to a finite population density within the tissues of the host. In the chemosymbiotic associations between members of the bivalve family Thyasiridae and their thiotrophic bacterial symbionts, hosts provide the mutualistic bacteria accommodated in the epithelial cells of their abfrontally expanded gill filaments with reduced sulphur species to fuel their autotrophic metabolism. Symbiotic thyasirids provide their symbiotic bacteria with the requisite reduced sulphur through the construction of extensive pedal tracts involved in sediment sulphide localization. Symbionts are acquired from a free-living pool of bacteria, and are periodically endocytosed and digested by host bacteriocytes. In this study, we demonstrate that unlike



the bacterial symbionts of many chemosymbiotic bivalves, the bacterial mutualists of *Thyasira flexuosa* and *Thyasira* cf. *gouldi* divide within the confines of host gill epithelial cells, possibly constituting a second method of symbiont renewal in thyasirid bivalves alongside environmental acquisition. Furthermore, exposure of *T. flexuosa* and *T.* cf. *gouldi* to elevated concentrations of thiosulphate, a reduced sulphur species used by many sulphur-oxidizing bacterial symbionts, results in the rapid onset of bacterial division and expansion of symbiont populations. Continued exposure to thiosulphate results in the unrestrained proliferation of bacterial symbionts and ultimately in the mortality of the host organisms. These results highlight the fragile and conditional nature of mutualistic outcomes in thyasirid bivalves, and may contribute to the explanation of the distinctively patchy distribution of symbiosis within this family and the possibility for the loss of symbiosis in some thyasirid lineages. Furthermore, the inability of thyasirid hosts to restrict bacterial division emphasizes the adaptive significance of behaviours such as sulphide mining and control over the transport and acquisition of sulphides in maintaining and controlling a stable population of bacterial mutualists.

## **Introduction**

Traditionally, symbioses between eukaryotic hosts and their microbial symbionts have been viewed in light of “mutualistic environment” models (Law & Lewis, 1983), a framework of ideas emphasizing direct cooperation between partners and the long-term evolutionary stability of mutualistic associations (Sachs *et al.*, 2011). This paradigm is being challenged and replaced by new research emphasizing symbioses as reciprocally

exploitative interactions, in which each of the partners involved seeks to maximize the benefits it obtains from its partner while providing a minimum of services and goods in return (Sachs *et al.*, 2011). Despite the ubiquitous and often stable nature of mutualisms seen in nature, the distinction between beneficial and parasitic interactions is being blurred by new research, and mutualisms are now recognized as being prone to reversion to autonomy and even parasitism (Sachs & Simms, 2006). Symbiotic associations are plagued by inherent conflicts of interests over issues such as resource exchange and symbiont transmission, raising the possibility of divergence in the evolutionary interests of the partners involved and the corruption of the system by cheating or exploitative symbionts (Douglas, 2008). For any symbiotic association to persist, the evolutionary interests of both partners must remain aligned (Herre *et al.*, 1999), and as such mechanisms must be put in place that ensure the proper exchange of resources, the suppression of would-be cheaters, and the assurance of repeated interactions between hosts and symbionts across generations (Douglas, 2008).

Mutualistic symbioses between marine invertebrates and chemoautotrophic bacteria are widespread both geographically and phylogenetically (Dubilier *et al.*, 2008), and often form an important ecological adaptation for survival in marginal environments such as hydrothermal vents and cold seeps (Chaston & Goodrich-Blair, 2010). Bacterial symbionts oxidize reduced chemical compounds to provide the energy for the fixation of inorganic carbon (Kleiner *et al.*, 2012), thus providing a valuable source of nutrients to their hosts. In turn, their invertebrate hosts display a suite of behaviours and adaptations enabling them to bridge the oxic/anoxic divide, providing their bacteria with both reduced chemical compounds (sulphides, methane etc.) and the electron acceptors (oxygen) they

require (Chaston & Goodrich-Blair 2010). In such nutritional symbioses, the hosts must ensure the existence of a population of bacterial symbionts sufficiently large to support their often extensive and sometimes exclusive reliance on symbiotically-derived organic carbon (Dando & Spiro, 1993; Caro *et al.*, 2009; Nyholm *et al.*, 2012). However, reliance of invertebrate hosts on their often large symbiont populations must be balanced by an ability to control the population size and dynamics of their bacterial partners, lest unchecked bacterial proliferation result in the overgrowth of host tissues (Neckelmann & Muscatine, 1983). In various chemosymbiotic associations, this may be accomplished through a variety of mechanisms, including the coupling of bacterial life cycles to a tightly regulated host cell death regime in *Riftia pachyptila* (Pflugfelder *et al.*, 2009), as well as the arrestment of bacterial division postulated for many chemosymbiotic bivalves (Stewart & Cavanaugh, 2006; Caro *et al.*, 2007; Kuwahara *et al.*, 2007).

The Thyasiridae is one of seven families of bivalves known to harbour symbiotic chemoautotrophic bacteria (Oliver & Taylor, 2012; Roeselers & Newton, 2012; Oliver *et al.*, 2013). In this group, gammaproteobacterial sulphur-oxidizing symbionts (Distel & Cavanaugh, 1994; Dufour *et al.*, 2014) are housed in enlarged extracellular spaces delineated by extensions of the cell membrane and microvilli of specialized gill epithelial cells known as bacteriocytes (Dufour, 2005). Bacterial symbionts of thyasirids are likely environmentally acquired (Dufour *et al.*, 2014), and are thought to be periodically digested by the host (Southward, 1986). Division of bacterial symbionts within the gill tissues of the host has not previously been documented, and the mechanisms of host control over symbiont population control and identity remain a mystery.

Not all members of the family host chemosynthetic bacterial symbionts, and the presence of bacterial symbionts in this group has been shown to vary on strikingly small phylogenetic scales (Southward, 1986; Batstone *et al.*, 2014). Furthermore, the relevance to the host of symbiotically derived resources varies based on relevant environmental parameters such as sulphide abundance (Dufour & Felbeck, 2006), and season (Donval *et al.*, 1989; Dando & Spiro, 1993). Symbiont identity is not conserved within the family (Rodrigues & Duperron, 2011) and within single species, hosts have the potential to associate with different bacterial phylotypes (Duperron *et al.*, 2012b). In thyasirids, the association between symbiotic thyasirids and the chemoautotrophic bacteria they harbour is regarded as facultative and relatively unstable (Batstone *et al.*, 2014). As such, this group has been presented as an example of symbiotic organisms in the early stages of the complex and highly coordinated symbioses seen in other groups of chemosymbiotic marine invertebrates (Duperron *et al.*, 2012a; Roeselers & Newton, 2012). Given the relatively novel and simplistic state of the symbiosis in thyasirid bivalves, these organisms may lack the complex mechanisms for symbiont population control seen in other groups of chemosymbiotic marine invertebrates.

It is possible that host control over the symbiont population in this group is related to behavioural adaptations, such as the extensive sub-sediment burrow construction involved in the localization of often sparse sulphide reserves (Dufour & Felbeck, 2003). Sulphides may then be transported to the host gills and thus their bacterial symbionts through the haemal transport of reduced sulphur, as has been documented in the vesicomyid bivalves (Arp *et al.*, 1984; Childress *et al.*, 1993) or through ventilation of the surrounding sediment and subsequent uptake of sulphide-laden water from deep within

the sediment (Dufour & Felbeck, 2003; Dando *et al.*, 2004). Control over such ventilation-based and/or haemal transport of sediment sulphides may allow thyasirids to control symbiont population through behavioural regulation of symbiont nutrient provision, though this possibility remains unexamined.

In this study, we sought to address the question of symbiont population regulation in the symbioses of *Thyasira cf. gouldi* and *Thyasira flexuosa*, two thyasirids known to harbour chemoautotrophic bacterial symbionts (Dando & Southward, 1986; Batstone *et al.*, 2014). Given the presumed simplicity of the symbiotic association in thyasirids and potential lack of the complex regulatory mechanism seen in other chemosymbiotic organisms (Duperron *et al.*, 2012a; Rodrigues & Duperron, 2012), this group provides an excellent opportunity to study host control over symbiont population dynamics and the maintenance of a mutually beneficial association in relatively unstable and vulnerable symbiotic associations. The aim of this study was to evaluate the role of the supply of reduced sulphur species to the bacterial symbionts of thyasirids in maintaining stable symbiont populations and regulating bacterial proliferation. The role of symbiont nutrient limitation in the regulation of symbiont life cycles and population size has been demonstrated in other symbiotic organisms such as the green hydra (Neckelmann & Muscatine, 1983) and the Red Sea coral (Muscatine *et al.*, 1989). However, the potentially crucial role of host control over sulphide supply has never been addressed in thyasirid bivalves, despite revelations that symbiont abundance may change in relation to experimentally controlled sediment sulphide concentrations (Dufour & Felbeck, 2006). We hypothesize that the ability of host chemosymbiotic organisms to control symbiotic sulphide exposure may constitute an important pillar in the regulation of bacterial

population dynamics in mutualistic associations between bivalves and their chemosynthetic bacterial symbionts.

## **Materials and Methods**

### **Sample collection**

#### *Thyasira flexuosa*

Sediment and clams were collected during the month of June 2013 in the Bay of Brest, immediately adjacent to the commercial port of the city of Brest, France. Sediment was collected using a dredge in 100 m long sampling transects. The sampling location (N 48°22.804, W 04°27.653) is characterized by organically enriched sediment due to runoff from the nearby city and surrounding agricultural lands (Jean & Hily, 1993).

*T. flexuosa* were isolated by sieving the sediment through a 1 mm mesh. Additionally, sediment collected at the same time and location was dry-sieved on a 1 mm mesh sieve. In order to maintain the health of the clams prior the beginning of the experiment, live thyasirids were placed at the surface of this sediment and allowed to burrow. Sediment microcosms, with burrowed thyasirids, were maintained in flow-through tanks at room temperature with a constant supply of ambient seawater from the Bay of Brest.

#### *Thyasira cf. gouldi*

Sediment was collected during the month of November 2013 from 2 sites in Bonne Bay (N 49°32. W 57°56.), a subarctic fjord located within Gros Morne National Park on the western coast of Newfoundland (Canada). Samples were collected from the

eastern arm of this bifurcated fjord, a sheltered, protected water mass due to the presence of a shallow sill separating it from the Gulf of St. Lawrence (Gilbert & Pettigrew, 1993). Sediment was collected at two sites: South East Arm, N 49°27.75, W 57°42.82 and Deer Arm, N 49°33.21, W 57°50.42. Both of these sites are characterized by muddy sediment highly enriched in organic matter due to the input of terrestrial organic matter and detritus.

Collected sediment was sieved on a 1mm mesh, and live clams were retrieved and immediately subjected to the experimental protocols and treatments.

### **Experimental design**

#### *T. flexuosa*

40 days after collection (and microcosm maintenance), clams were once again removed from the sediment through the process of sieving. Twenty clams were placed in beakers containing 0.2 µm filtered, heat-sterilized seawater from the Bay of Brest. Ten of the clams were used to establish a control for symbiont morphology and abundance throughout the experimental procedure. These control specimens were maintained in beakers with filtered, sterilized seawater.

To investigate the effect of elevated thiosulphate (a reduced sulphur species that can be utilized by many chemoautotrophic bacterial symbionts (Kleiner et al, 2012)) concentrations, 10 clams were placed in a beaker containing a solution of 1% sodium thiosulphate in filtered, sterilized seawater. On the 2<sup>nd</sup> and 4<sup>th</sup> days of the experiment, 0.5 g of thiosulphate was added to the experimental treatment to replenish the supply of thiosulphate as it spontaneously oxidizes at room temperature and is used by the bacterial

symbionts. The beakers were kept at room temperature to reproduce the temperature, similar to conditions in the Bay of Brest during the month of August (roughly 18°C, Paillard, pers. comm.).

Prior to the initiation of the experiment, 4 clams were dissected to establish a baseline for the characteristics and morphology of both host cells and symbiotic bacteria. Gills were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer for 4 hours before being transferred to a 0.1 M sodium cacodylate buffer for storage. Subsequently, 2 clams from both the control (sterilized, filtered seawater) and experimental (1% thiosulphate in sterilized, filtered seawater) treatments were removed and dissected every 3 days. Gills were fixed as described above.

Additionally, thysirids in both the thiosulphate-enriched and control treatments were examined on a daily basis to determine the state of the clams and estimate thysirid survival based on externally visible characteristics. Clams were determined to be dead if their valves were open. The colour and appearance of the thysirids' gills were also noted, both through the transparent shells of the bivalves whilst still alive and upon dissection. Dead clams found in the thiosulphate treatment were also dissected and their gills fixed for transmission electron microscopy as described above.

#### *T. cf. gouldi*

64 individuals of *T. cf. gouldi* were placed in beakers containing 0.2 µm filter-sterilized seawater from Bonne Bay, Newfoundland. Control specimens (n = 16) were maintained in beakers containing seawater with no added chemicals, while the other 48 specimens were placed in beakers containing 2%, 1% or 0.5% dissolved sodium



thiosulphate (16 per treatment). These beakers were kept at 4°C to replicate the typical temperature of seawater at the benthic interface of Bonne Bay, NL during the month of November (Dufour, pers. comm.).

At the beginning of the experiment, 4 clams (not taken from the control and experimental treatments described above) were dissected to establish a baseline representation of bacterial symbiont and host cell morphology. Gills were fixed as described for the gills of *T. flexuosa*. Subsequently, clams from the control and various experimental treatments were removed at selected times after the initiation of the experiment (12 hours, 1 day, 2 days, and 4 days). At each of these sampling times, individuals from each treatment were randomly selected and removed for the purpose of gill fixation as described above. Unlike in the experiment conducted on *T. flexuosa*, no supplementary thiosulphate was added to the experimental treatments over the course of the experiment, as it was assumed that the rate of thiosulphate decay and utilization by symbionts would be reduced at the lower temperature used in the *T. cf. gouldi* experiment (4°C compared to 18°C for the *T. flexuosa* trials).

### **Transmission electron microscopy**

The fixed gills from both species of thyasirids were stained in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer for 1 hour and embedded in an EPON resin following dehydration in an increasing ethanol series. Subsequently, ultra-thin sections (~60-70 nm) were cut on an ultramicrotome and placed on copper TEM grids. These were post-stained with uranyl acetate and lead citrate and examined using a Philips 300

transmission electron microscope at the Electron Microscopy and Flow Cytometry Unit of the Health Sciences Centre (Memorial University of Newfoundland and Labrador).

For both the *T. cf. gouldi* and *T. flexuosa* experiments, 2 of the individuals collected at the beginning of the experiment as baseline specimens were examined. Further specimens, from both control and experimental treatments, were also investigated and photographed under the transmission electron microscope. The number of individuals examined from each species, treatment, and sampling time is listed in Table 1.

**Table 1.** Number of thyasirids examined under the transmission electron microscope for the experiments on both species at various elapsed times. Sample sizes are listed for control specimens (maintained in filtered seawater) and experimental specimens kept in the presence of dissolved thiosulphate (TS). The examination of deceased individuals is indicated by the presence of an asterisk (\*) after the sample size.

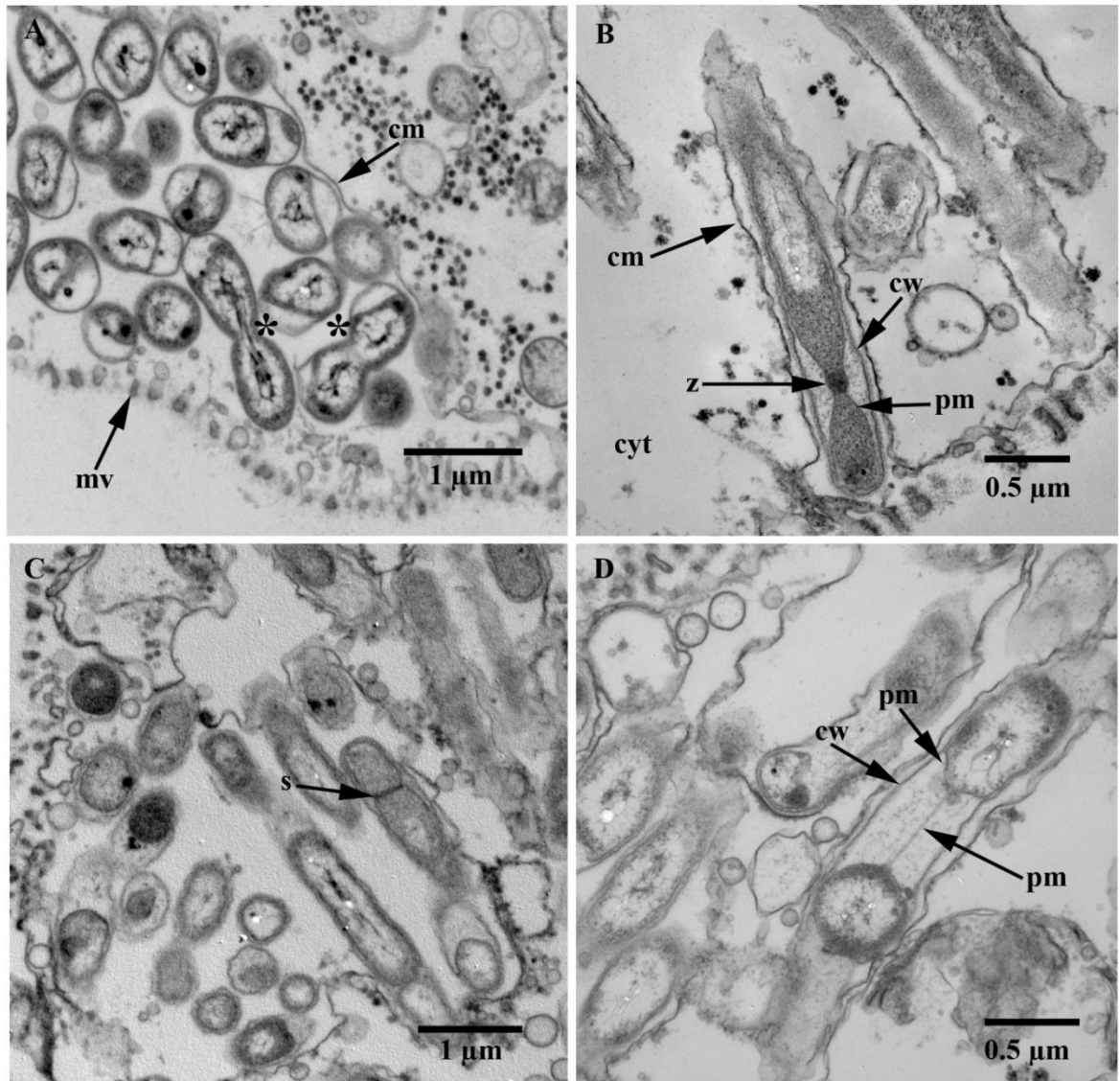
<i>Thyasira cf. gouldi</i>					<i>Thyasira flexuosa</i>		
Time	Control	0.5% TS	1% TS	2% TS	Time	Control	TS
12 h	1	1	1	2	3 d	2	2
1 d	1	1	1	2	6 d	2	2+2*
2 d	1	1	1	2	9 d	2	n/a
4 d	1	1	1	2	12 d	2	n/a

For each individual examined, the frontal-most 2 bacteriocytes of at least 5 host gill filaments were examined at various magnifications to view both host and symbiont cell characteristics. The symbionts of these cells were closely examined for the presence of clear morphological indicators of cell division, and images were taken to capture host cell and bacterial symbiont morphology.

## Results

The gill structure and general cellular characteristics of *T. cf. gouldi* and *T. flexuosa* were consistent with previous descriptions of symbiotic thyasirid species. As is typical for symbiotic thyasirids having type 3 gills, abundant symbionts were maintained extracellularly, accommodated by the host in evaginations formed by extensions of the host cell membrane and cytoplasm (Dufour, 2005).

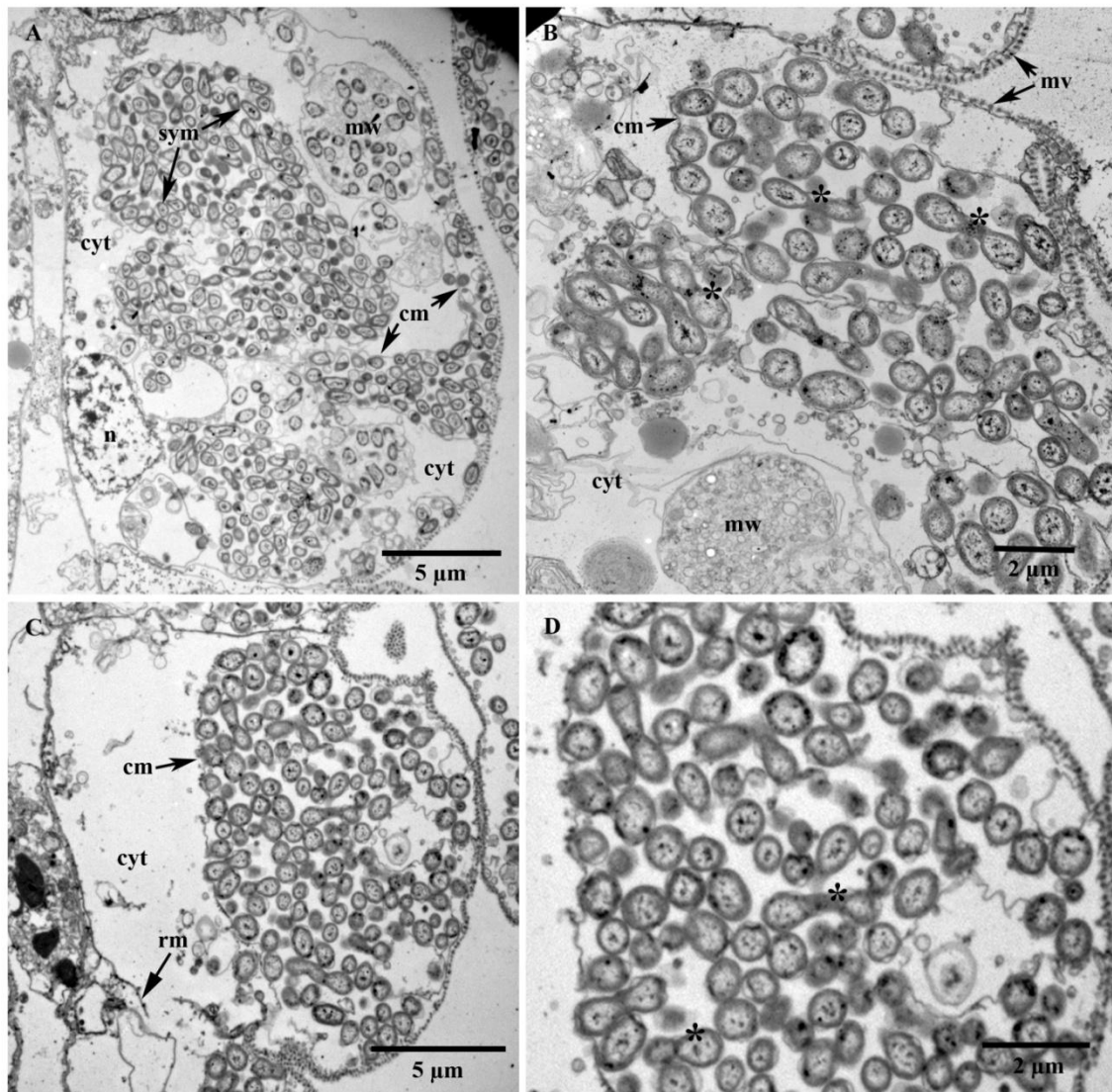
In both *T. cf. gouldi* and *T. flexuosa*, the presence of dividing bacterial symbionts within the extracellular area proffered by the host was observed. Symbiont division was inferred based on characteristic patterns in their morphology, including evident “pinching” involved in the ingrowth of the plasma membrane and cell wall (Fig 1A,B). In some cases, dividing bacterial cells displayed a distinct electron dense structure at the site of plasma membrane pinching and future cell separation, possibly indicating the presence of an intact Z ring (Fig 1B). In other dividing symbiotic cells, a linear structure appearing to be a fully formed septum can be seen separating two daughter cells (Fig 1C), while in some fully divided bacterial cells, a thin connective thread of plasma membrane seemed to link the two daughter cells despite their sharing a cell wall (Fig 1D). The presence and relative abundance of dividing symbiotic bacteria varied between the treatments to which the hosts were subjected, as described below.



**Figure 1.** Transmission electron micrographs of *Thyasira flexuosa* and *T. cf. gouldi* symbionts. (A) Dividing bacterial symbionts (\*) in the bacteriocytes of a freshly collected *T. flexuosa*. Symbionts are located in an extracellular pocket delineated by the microvillar (mv) band and cell membrane (cm) of the host cell. (B-D) Bacterial symbionts of *T. cf. gouldi* maintained in 2% thiosulphate-enriched seawater. (B) A dividing symbiont with a putative complete Z ring (z) separating 2 daughter cells. The separating plasma membranes (pm) of the daughter cells are visible, along with their shared cell wall (cw). The symbiont is located within an invagination of the host cell membrane and cytoplasm (cyt). (C) A bacterial symbiont can be seen in the late stages of cell division, with a complete septum (s) separating two daughter cells. (D) A dividing bacterial symbiont is seen in the late stages of cell division. Two daughter cells, still sharing the same cell wall, are separated by a thin thread of plasma membrane.

### *Thyasira flexuosa*

Symbiotic bacteria were abundant in the bacteriocytes of *T. flexuosa*, as observed in baseline specimens, control specimens maintained in filtered seawater, and specimens exposed to elevated concentrations of thiosulphate (Fig 2). Some bacterial symbionts were seen to be undergoing cell division in the bacteriocytes of specimens regardless of whether or not they had been exposed to thiosulphate-enriched seawater. Dividing bacteria were present in clams dissected upon collection, after maintenance in sediment, and when held in the absence of thiosulphate (Fig 1A). However, under these conditions, bacterial cell division was rare, and the majority of host bacteriocytes lacked visibly dividing bacterial symbionts (Fig 2A). In contrast, the bacteriocytes of *T. flexuosa* exposed to the thiosulphate-enriched seawater contained a much larger proportion of bacterial symbionts undergoing cell division. After 3 days of maintenance in seawater artificially enriched in thiosulphate, bacterial division was seen much more frequently with all individuals (n = 6) examined possessing dividing bacterial symbionts in all bacteriocytes examined (n = 60). Additionally, the bacteriocytes contained an exceptionally high number of dividing bacteria (Fig 2B-D).



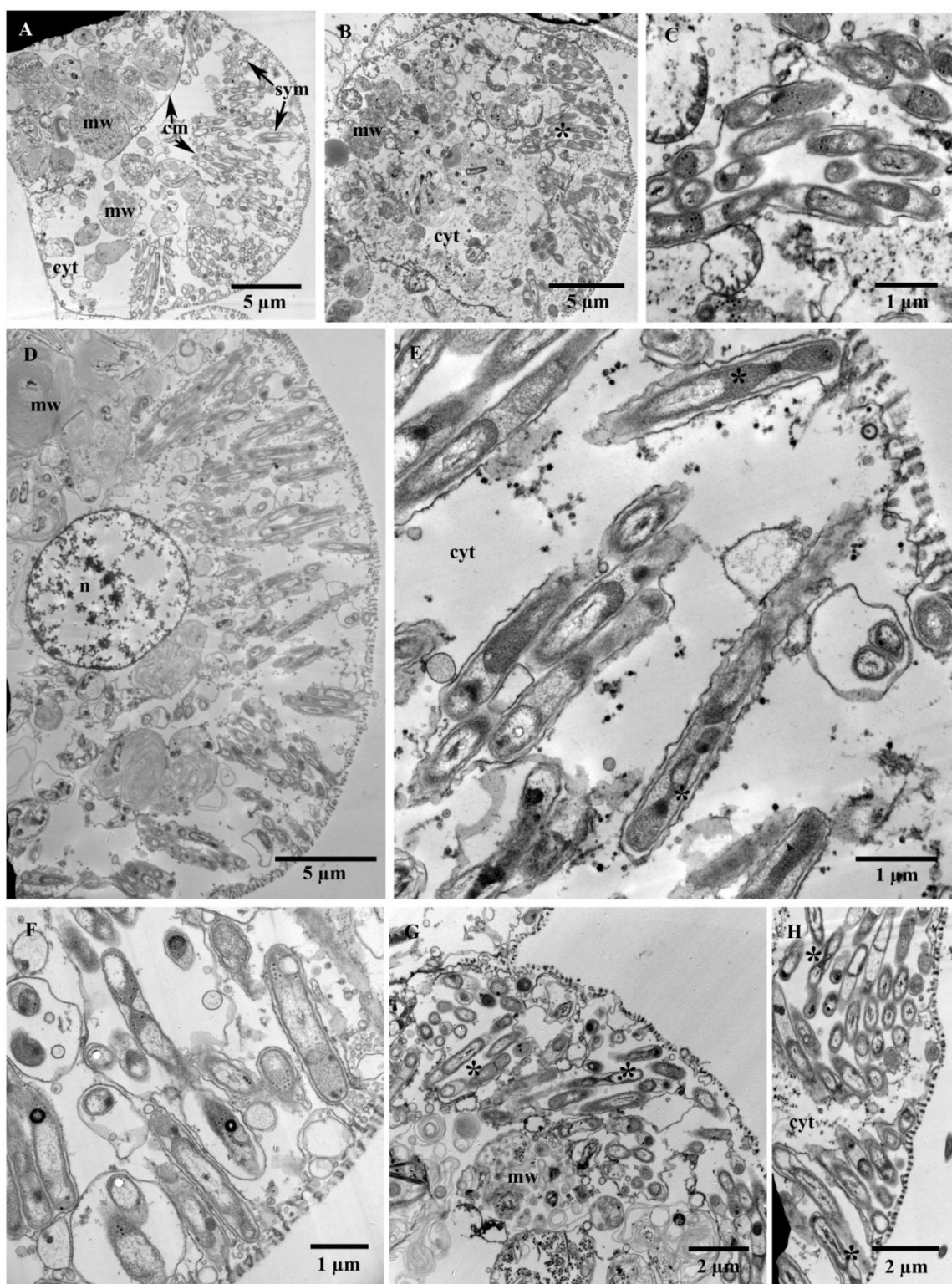
**Figure 2.** Transmission electron micrographs of *Thyasira flexuosa* bacteriocytes. (A) Bacteriocyte of a freshly collected (baseline) individual. Bacterial symbionts (sym) are seen in the extracellular space, surrounded by extensions of the host cell membrane (cm) and cytoplasm (cyt). Membrane whorls (mw), concentric rings of degraded bacterial cell walls and a nucleus (n) are visible within the host cell. (B-D) Bacteriocytes of specimens exposed to thiosulphate-enriched seawater for 3 days. (B) Dividing bacterial symbionts (indicated by an asterix, \*) are located within the extracellular space enclosed by the host cell membrane. Microvilli (mv) are visible on the external surface. (C) Dividing bacterial symbionts, located extracellularly. The cell membrane of the host features a partially ruptured membrane (rm). (D) High magnification of the symbionts seen in (C). Numerous bacterial symbionts are dividing (\*).

### ***Thyasira cf. gouldi***

Similar to the condition observed in *T. flexuosa*, the bacteriocytes of *T. cf. gouldi* maintained in the absence of added thiosulphate generally lacked dividing symbionts (Fig 3A). We can however confirm that in some cases, the bacterial symbionts of *T. cf. gouldi* undergo cell division within the extracellular spaces of host bacteriocytes (Fig 3B,C). However, the majority of host bacteriocytes lacked dividing bacteria and, when observed, symbiont division was quite limited (Fig 3C).

Exposure to 2% thiosulphate resulted in a rapid and remarkable increase in the frequency of bacterial division after as little as 12 hours. In the bacteriocytes of *T. cf. gouldi* exposed to 2% thiosulphate for 12 h, bacterial division was rampant, with large proportions of bacterial symbionts undergoing cell division (Fig 3D-F). The proportion of bacterial cells undergoing division within a given host bacteriocyte, as well as the frequency of host bacteriocytes containing one or more dividing symbionts were substantially higher in individuals maintained in the presence of thiosulphate.

**Figure 3 (Next Page).** Transmission electron micrographs of *Thyasira cf. gouldi* bacteriocytes and symbionts. (A) Bacteriocyte of a control specimen after 12 h of elapsed time. Symbionts (sym), none of which are undergoing cell division, are seen extracellularly, bounded by the host cell membrane (cm). Within the host cell cytoplasm (cyt), membrane whorls (mw) are visible. (B) Bacteriocyte of a freshly collected (baseline) individual. Dividing bacterial symbionts (\*) are seen in the extracellular space. (C) Close-up of (B), showing bacteria dividing. (D-F) Bacteriocytes of specimens maintained for 12 h in 2% thiosulphate. (D) Bacterial symbionts can be seen undergoing cell division in the extracellular space of the host cell. (E) High magnification micrograph of bacterial symbionts undergoing cell division in the bacteriocytes seen in (D). (F) High resolution micrograph illustrating the presence of dividing bacterial symbionts. (G) Bacteriocyte of a specimen maintained in 2% thiosulphate for 24 h. Dividing bacterial symbionts are present in the extracellular symbiont population. (H) Bacteriocyte of an individual kept in 1% thiosulphate for 24 h. Dividing bacterial symbionts can be seen.





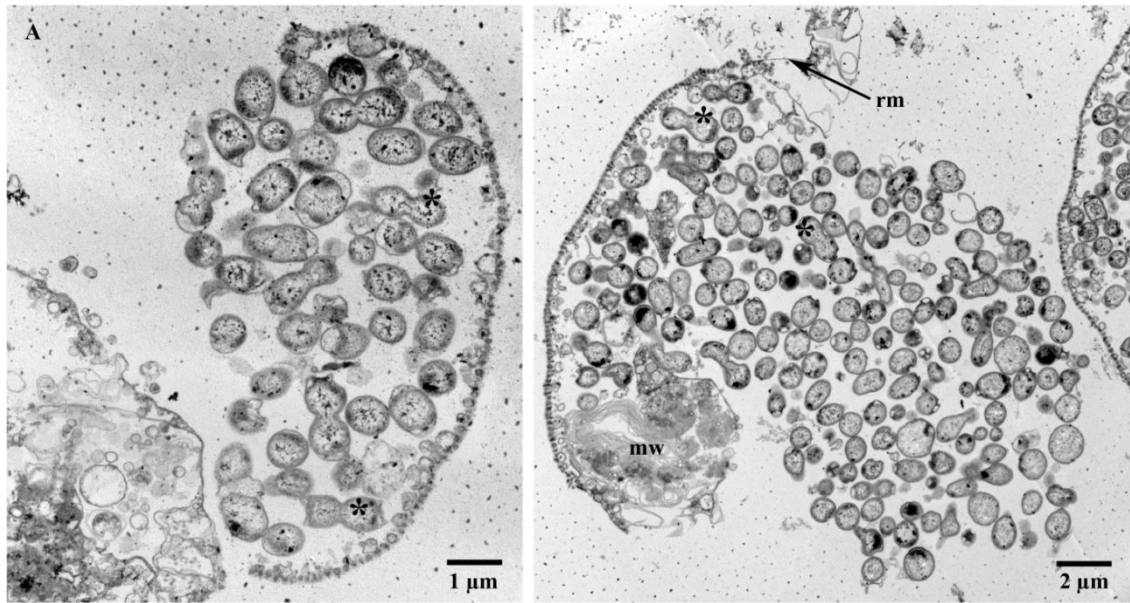
Symbiont division continued to be observed following the initial sampling time of 12 h. Bacterial division remained frequent after 24 hours exposure to 2% thiosulphate (Fig 3G) and after 2 and 4 days of exposure to thiosulphate-enriched seawater, though the frequency of dividing bacterial symbionts may have declined with time given that thiosulphate supply was not replenished. Similarly, symbiont division was observed under both 1% thiosulphate (Fig 3H) and 0.5% thiosulphate treatments, though the frequency of bacterial division was noticeably lower, especially under the lowest concentration (0.5%) used.

#### **Death of bacteriocytes and host mortality**

During the *T. flexuosa* experiment, a high degree of host mortality was observed in the thiosulphate exposure treatment, but not in the control treatment. Of the 10 clams placed in thiosulphate-enriched seawater, 2 were dissected 3 days into the experiment. On the sixth day of the experiment, only 2 of the remaining 8 bivalves were alive. The other 6 (constituting a mortality rate of 75% after 6 days) were characterized by gaping valves and deteriorating tissues. In these individuals, white masses were observed along the surface of the gill filaments and loose in the mantle cavity. This abnormal morphology was associated with a noticeable change in gill filament ultrastructure, with gills appearing to be undergoing degradation. However, this morphology was not simply a feature of host mortality, as it was also observed in living individuals (with closed valves) after 4 days of maintenance in the thiosulphate treatment (at which point no bivalve mortality had yet been noted). The gills of these specimens had a whitish appearance (as opposed to the typical rose-red pigmentation), readily apparent through the transparent shells of the clams. In fact, the white masses described in deceased bivalves were also

observed in the 4 live individuals dissected after both 3 and 6 days of maintenance in thiosulphate-rich seawater. In all these live individuals, gills appeared to be covered by white masses, some of which were loosely attached or floating in the mantle cavity. This was associated with an observed decrease in gill filament integrity and condition.

The gills of the living *T. flexuosa* specimens held in thiosulphate-enriched seawater, as detailed above, were characterized by a large proportion of dividing bacterial symbionts. Additionally, these clams contained bacteriocytes that appeared to be ruptured or detached from the basal lamina of gill epithelia. Other bacteriocytes appeared to have marginally compromised cell membranes, possibly indicating cell rupture (Fig 2C). However, the presence and abundance of loose bacteriocytes, untethered from host basal laminae or loosely attached to the gill filaments was most noticeable in deceased bivalves, dissected after 6 days of exposure to elevated thiosulphate concentrations (Fig 4). In these individuals, masses of bacteria were found in association with certain host cell features such as the microvillar band that typically encloses the extracellular space in which bacterial symbionts reside in thysirids (Fig 4A,B). However, this mass of bacteria and microvillar band was no longer associated with intact host bacteriocytes. In these masses of bacteria liberated by ruptured bacteriocytes, the proportion of dividing symbionts was high, indicating that the death of host bacteriocytes may be due to an unsustainably high level of bacterial division or symbiont population size. As previously mentioned, the onset of this highly atypical gill morphology and bacteriocyte condition was associated with significant host mortality (75% after 6 days). In contrast, all of the 8 control individuals were alive after 6 days of experimentation (0% mortality) and none died until the 11<sup>th</sup> day of the experiment.



**Figure 4.** Transmission electron micrographs of ruptured *Thyasira flexuosa* bacteriocytes, seen in deceased individuals collected after 6 days of maintenance in thiosulphate-enriched seawater. (A) Dividing bacterial symbionts (\*) are present but no longer confined by host cell extensions, and are seen in loose aggregations, both inside and outside liberated host bacteriocytes. (B) Dividing bacterial symbionts are seen. Remnants of a once-intact host bacteriocytes, including membrane whorls (mw) and a ruptured cell membrane (rm) are seen. Bacteria and the associated host cell features are loose within the mantle cavity and are no longer tethered to the basal lamina of the gill epithelium.

No host mortality or ruptured bacteriocytes were observed in the *T. cf. gouldi* experiment, which was conducted at a lower temperature (4°C) and was carried out without the renewal of sodium thiosulphate described for the experimental treatment of *T. flexuosa*.

## **Discussion**

### **Division of bacterial symbionts**

In mutualistic symbioses between thiotrophic bacteria and bivalve hosts, hosts must be able to acquire viable symbionts and maintain bacterial populations sufficient to meet the nutritional demands of the host organism (Roeselers & Newton, 2012). The mechanisms of this acquisition vary greatly between the chemosymbiotic bivalve families, a fact that exerts profound ecological and evolutionary influence upon the nature of their symbiotic associations. In some bivalve families, such as the Solemyidae (Krueger *et al.*, 1996) and Vesicomysidae (Cary & Giovanni, 1993), bacterial symbionts are passed on to each new generation of host through vertical transmission of symbionts through the maternal germ line (Krueger *et al.*, 1996), leading to extensive co-speciation and co-adaptation between the host and symbiont. Such associations are often obligate, (Kuwahara *et al.*, 2007) with host relying extensively or exclusively on symbiotically derived organic carbon (Stewart & Cavanaugh, 2006). In the Solemyidae, the large symbiont populations required to meet nearly all of the hosts' metabolic demands are established by transovarian inheritance and subsequent expansion of symbiont populations in the gills of juveniles (Krueger *et al.*, 1996). As nutrient transfer between symbionts and host occurs through the direct transfer (translocation) of metabolites rather than the digestion of cells (Fisher & Childress, 1986), symbionts do not regularly divide in adult clams (Steward & Cavanaugh, 2006; Kuwahara *et al.*, 2007), and symbiont populations are stable throughout adulthood.

In other well-studied groups of chemosymbiotic organisms such as the lucinid bivalves (Gros *et al.*, 1996; Brissac *et al.*, 2011) and the deep-sea vestimentiferan tubeworms of the genus *Riftia* (Nyholm *et al.*, 2012), symbionts are acquired from a free-living pool of bacteria. In order for newly-symbiotic bacteria to establish a sizeable and mature symbiont population, bacterial cells must either divide within host tissues, as is the case of *Riftia pachyptila* (Nussbaumer *et al.*, 2006) or be continuously re-acquired throughout the host life cycle, as has been documented for the lucinid bivalve *Codakia orbiculata* (Gros *et al.*, 2012).

In the lucinid bivalves, hosts are often incredibly reliant on the chemoautotrophic metabolism of their symbionts, which may provide most of the host organisms' nutrition. Symbionts occupy up to 33% of host gill tissue area (Caro *et al.*, 2009), a figure similar to that observed for the trophosome of *Riftia pachyptila* (Bright & Sorgo, 2003), a species that lacks a gut and mouth and is completely dependent on the energetic input from its symbiont population (Nyholm *et al.*, 2012). In lucinids, transfer of nutrients from symbiont to host occurs through lysosomal digestion of bacterial symbionts (Liberge *et al.*, 2001; Caro *et al.*, 2009), and digested symbionts are replaced by the acquisition of new free-living bacterial symbionts in the surrounding environment (Gros *et al.*, 2012). Division of bacterial symbionts within host gill tissues is not postulated to be a mechanism for the renewal of bacterial symbiont populations, as dividing bacterial cells are rarely seen in lucinid bivalves (Caro *et al.*, 2007). Nonetheless, the evidence for the reliance of lucinid bivalves on the acquisition of novel symbionts to replace digested bacteria is difficult to reconcile with studies that demonstrate strikingly low numbers of free-living symbiotic bacteria in the hosts' environment (Gros *et al.*, 2003). Symbionts

may be so rare that they constitute less than 0.3% of bacterial cells in the *Thalassia testudinum* seagrass meadows home to many lucinid species, indicating that this environmental reservoir may be insufficient to constantly renew lucinid symbiont populations (Green-Garcia & Engel, 2012).

The symbiosis between thyasirid bivalves and thiotrophic bacteria is often regarded to be one of the least derived symbiotic relationships observed amongst the families of chemosymbiotic bivalves (Roeselers & Newton, 2012). Within the family, asymbiotic and symbiotic species exist over small phylogenetic scales (Southward, 1986; Batstone *et al.*, 2014), and symbiont identity is variable both between (Rodrigues & Duperron, 2011) and within (Duperron *et al.*, 2012b) species. Nonetheless, many symbiotic thyasirids rely extensively on the input of symbiotically derived metabolites, which in some species accounts for over 50% of host carbon supply (Spiro *et al.*, 1986; Dando & Spiro, 1993). As in the lucinid clams, transfer of nutrients from symbiont to host is thought to occur on the basis of endocytosis and lysis of bacterial cells (Southward, 1986; Le Pennec *et al.*, 1988a), and symbionts are thought to be acquired from a free-living pool of bacterial symbionts (Rodrigues & Duperron, 2011). Based on a nutrient transfer model of strict reliance of symbiont endocytosis and lysosomal digestion, it is difficult to reconcile the striking degree of host dependence on symbiotically-derived organic carbon (Spiro *et al.*, 1986) with a system of symbiont population renewal similar to the lifelong re-acquisition of symbionts seen in the lucinid bivalve *Codakia orbiculata* (Gros *et al.*, 2012).

As such, the discovery of dividing bacterial symbionts in the bacteriocytes of both *Thyasira* cf. *gouldi* and *T. flexuosa* indicates that the replacement of digested symbiotic

bacteria may occur not only through the acquisition of new symbionts from their environment, but also from limited symbiont division within host tissues. Bacterial division in the symbiont population of both *T. flexuosa* and *T. cf. gouldi* occurred in specimens collected directly from the field, without further manipulation and exposure to increased thiosulphate concentrations. Features of cell division included the ingrowth of the cytoplasmic membrane and cell wall (Fig 1A), as well as the presence of a Z ring (Fig 1B), the constriction of which is postulated to cause the ingrowth of the cell envelope (Weiss, 2004). In some specimens, daughter cells were separated by fully formed septa (Veiga & Pinho, 2012) and threads of the cytoplasmic membrane following separation (Fig 1C,D). Though limited under natural conditions, the discovery of dividing bacterial symbionts helps explain the incongruency between the extensive degree of nutritional reliance on the metabolic activity of symbionts seen in many thyasirid bivalves (Spiro *et al.*, 1986; Dando & Spiro, 1993) and the need to locate free-living bacterial symbionts in reduced sediments (Green-Garcia & Engel, 2012).

### **Thiosulphate exposure and symbiont division**

While dividing symbionts were observed in the bacteriocytes of *T. flexuosa* (Fig 1A) and *T. cf. gouldi* (Fig 3B,C), a much higher proportion of bacterial cells were dividing after exposure to thiosulphate-enriched seawater. In *T. flexuosa*, 3 days of exposure to thiosulphate-rich seawater resulted in the presence of dividing symbiotic bacteria within all of the bacteriocytes (10 per specimen) examined. In addition, the relative abundance of dividing bacterial symbionts was noticeably higher in these individuals (Fig 2B-D). Further investigations of the *T. cf. gouldi* symbiosis revealed that the initiation of bacterial division occurs remarkably rapidly after exposure to 2%

thiosulphate in seawater, with host bacteriocytes containing numerous symbionts in the process of cell division after just 12 hours of exposure (Fig 3D-F). Exposure of *T. cf. gouldi*, and their symbiotic bacteria to lesser concentrations of thiosulphate (1%, 0.5%) also resulted in the detection of symbiont division greater than that observed in samples dissected and fixed immediately after field collection (Fig 3A,G), though there was noticeably less dividing bacterial symbionts in the gills of host bivalves exposed to the lowest (0.5%) thiosulphate concentration.

For mutualistic symbioses to persist over evolutionary timeframes, congruence of the evolutionary interests of both the host and symbionts is essential (Herre *et al.*, 1999). As such, the host must be able to ensure not only that it is obtaining the nutrients it requires from its symbiotic partners and is suppressing cheating symbiont phylotypes (Douglas, 2008), but also that its symbionts are not proliferating too rapidly. The relative abundance of symbionts to the host cells that accommodate them must remain fairly constant over short time frames, highlighting the importance of host regulation over the population size and dynamics of its partners (Neckelmann & Muscatine, 1983). The challenge of sustaining an enriched symbiont microhabitat that supports and maintains the crucial and often staggering levels of symbiotic carbon fixation without leading to the exponential growth of bacterial symbiont populations is one that all chemosymbiotic organisms must resolve (Caro *et al.*, 2007).

The symbionts of the vestimentiferan tubeworm *Riftia pachyptila* divide regularly within the host trophosome to replenish the population of thiotrophic bacteria available to the host (Nussbaumer *et al.*, 2006). Nevertheless, the population of symbiotic bacteria is kept in check by a tightly controlled cycle of host tissue proliferation and apoptosis. In *R.*



*pachyptila*, totipotent bacteriocyte stem cells migrate outwards from the centre of trophosome lobules, and undergo apoptosis in the degenerating region near the external surface of the lobules. Symbiotic bacteria are lysed alongside the apoptosis of degenerating bacteriocytes (Pflugfelder *et al.*, 2009), effectively preventing the symbiont population from expanding too rapidly and overcoming the tissues of their host.

In chemosymbiotic associations between bivalves and sulphur oxidizing bacteria, the control of bacterial population size frequently relates to the direct suppression of bacterial division. In the lucinid bivalve *Codakia orbicularis*, symbiont division is seemingly restricted and prevented by the host, as evidenced by the characteristic inclusion of multiple genome copies within symbiotic bacteria (Caro *et al.*, 2007; Caro *et al.*, 2009). This multigenomic state is frequently interpreted as evidence of fast growing bacteria (Caro *et al.*, 2007), or cells that are capable of rapid division should adequate nutrients become available (Thorsen *et al.*, 1992). The presence of bacterial symbionts with multiple genome copies exhibiting little or no division has also been observed in solemyid clams (Stewart & Cavanaugh, 2006), suggesting the possibility of a similar mechanism of host repression of symbiont division. In the vertically transmitted symbionts of the vesicomyid bivalve *Calyptogena okutanii*, sequencing of the symbiont genome has found it to lack *FtsZ*, a gene crucial for cytokinesis and cell division. As such, this highly adapted, vertically transmitted symbiosis characterized by extensive co-speciation may be an example of a host exerting complete control over the dynamics of population expansion of its bacterial symbionts (Kuwahara *et al.*, 2007). These families of bivalves are thought to represent more highly adapted chemosymbiotic relationships in comparison to the Thyasiridae (Roeselers & Newton, 2012), and seem to have evolved

mechanisms to directly control the potentially dangerous proliferation of bacterial symbionts in their tissues whilst hosting symbiont population sufficient in size to meet their metabolic demands.

Direct suppression of bacterial division likely does not occur in the Thyasiridae, as indicated by crucial differences in symbiont morphology and physiology. In lucinid symbioses, the environmentally occurring, free-living form is noticeably smaller than that observed in host bacteriocytes. Within host gill tissue, cells can increase by up to 5 times in size, and are frequently found to be as large as 5  $\mu\text{m}$  in length. This may be related to the deregulation of symbiont growth within host cells (Gros *et al.*, 2003), and potentially the multi-genomic state characteristic of the division-inhibited bacterial symbionts of chemosymbiotic bivalves (Caro *et al.*, 2007). In comparison, the size of thyasirid bacterial symbionts is substantially lower (Le Pennec *et al.*, 1988b), and there is no evidence for the presence of multiple genome copies in thyasirid symbionts to date. Indeed, the symbionts of several thyasirid species, including *T. flexuosa* are consistent in size with the environmental, free-living stage of the closely related lucinid symbionts (Gros *et al.*, 2003), with which they are closely related (Rodrigues & Duperron, 2011). This raises the possibility that similar free-living bacterial symbionts undergo very different treatments in the gills of lucinid and thyasirid bivalves. In the Lucinidae, growth of bacterial cells may be deregulated whilst division is arrested, resulting in the characteristically large and multi-genomic bacterial state (Caro *et al.*, 2007; Caro *et al.*, 2009). This process does not appear to occur in the Thyasiridae, potentially indicating an inability of the host to suppress bacterial division directly. The rapid expansion of the bacterial symbiont populations of *T. cf. gouldi* and *T. flexuosa* when exposed to thiosulphate, indicate that

the hosts are indeed likely incapable of restricting bacterial division through cellular control mechanisms such as the production of bacteriostatic compounds postulated for *Codakia orbicularis* (Caro *et al.*, 2007).

Bacterial population expansion following exposure of their thiasirid hosts' to increased thiosulphate concentrations indicates that the supply of inorganic nutrients plays an important role in determining and controlling bacterial symbiont population dynamics in these symbioses. Similar results have been reported for the symbioses between the green hydra *Hydra viridis* and its photosynthetic algal symbionts *Chlorella* (Neckelmann & Muscatine, 1983) as well as the association between the Red Sea coral *Stylophora pistillata* and its symbiotic zooxanthellae (Muscatine *et al.*, 1989). In the *H. viridis*-*Chlorella* symbiosis, enrichment of the hydra culture media with a mixture of nitrate, phosphate and sulphate results in a highly enhanced growth rate of the symbiotic algae, ultimately resulting in the overgrowth of the host cells of *H. viridis* (Muscatine & Neckelmann, 1981; Neckelmann & Muscatine, 1983). Crucially, while slightly enlarged symbiont populations can be restrained via digestion and expulsion of algal symbionts (Neckelmann & Muscatine, 1983), extensive nutrient enrichment results in the overgrowth of host cells and eventually the death of the host organism itself (Muscatine & Neckelmann, 1981). In the *Stylophora pistillata*-zooxanthellae symbiosis, addition of a nitrogen source (ammonium), results in the approximate doubling of symbiont population size over 14 days, but does not result in the death of its host (Muscatine *et al.*, 1989). Nonetheless, expansion of the symbiont population results in a reduction in carbon translocation from symbionts to host, implying a fitness cost associated with nutrient excess and subsequent symbiont population expansion (Hoegh-Guldberg & Hinde, 1986).

In *T. cf. gouldi* and *T. flexuosa*, excess supply of thiosulphate, a source of reduced sulphur resulted in the rapid expansion of the symbiotic bacterial population. In *T. flexuosa*, continued addition of thiosulphate resulted in severely deleterious effects on the host bivalves. Notably, this resulted in 75% mortality after 6 days of exposure to the thiosulphate-rich seawater, along with the appearance of ruptured bacteriocytes and degradation of the gill ultrastructure. TEM analysis revealed these white agglomerations to be ruptured bacteriocytes full of dividing bacterial symbionts (Fig 4). The mortality of the host in response to exposure to elevated thiosulphate concentrations further illustrates the inability of thyasirid bivalves to restrain symbiont division. While it may be argued that the deterioration of *T. flexuosa* bacteriocytes and eventual host mortality may have been caused by thiosulphate poisoning rather than the rapid proliferation of bacterial symbionts, no host mortality or ruptured bacteriocytes were observed in the *T. cf. gouldi* experiment, despite their exposure to twice the concentration of the initial thiosulphate levels used in the *T. flexuosa* experiment. This discrepancy is likely explained by a difference in the ambient temperature at which the experiments were conducted (4°C for *T. cf. gouldi*, 18°C for *T. flexuosa*), in turn resulting in a drastic differential in the rate of bacterial symbiont division. As such, bacteriocyte degeneration and host mortality is best explained by the uncontrolled proliferation of bacterial symbionts, highlighting the importance of host regulation of the chemical composition of the fluid within the mantle cavity (in which bacterial symbionts are bathed). This likely constitutes a crucial adaptation to controlling the population of bacterial symbionts and preventing a highly deleterious outcome for the host organism.

Though an understanding of the mechanisms regarding the supply of reduced sulphur species is beyond the scope of this study, previous research may shed light on how thyasirid bivalves may potentially control the supply of reduced sulphur species to their bacterial symbionts. Thyasirid bivalves are notable in the construction of burrows in the sediment with their highly extensible foot, which can extend up to 30 times its length in the interest of accessing and mining pockets of sulphide deep within the sediment (Dufour & Felbeck, 2003). The ability to access these reserves of reduced sulphur allows them to obtain patchily distributed sulphide deposits within the sediment, and is a crucial adaptation to the acquisition of reduced sulphur by the host organism (Dufour & Felbeck, 2003). In the vesicomyid clams *Calymene magnifica* and *C. elongata*, sulphide transport occurs from similar sub-surface burrows through the use of serum-born sulphide-binding proteins. Sulphides diffuse across the epithelium of the foot and are then transported to the gills where they are used by bacterial symbionts (Arp *et al.*, 1984; Childress *et al.*, 1993). A similar mechanism has been postulated for the thyasirid bivalves (Dufour & Felbeck, 2003). Thyasirid bivalves also possess a posterior inhalant aperture, that draws water deep from the sediment in association with the ventilation of their sub-surface burrows (Allen, 1958; Dando *et al.*, 2004), and it is also possible that sulphides are directly drawn into the mantle cavity in order to supply the symbionts with their requisite compounds. Thyasirid bioirrigation results in the oxidation of surrounding sediments and the dissolution of iron sulphides, leading to the extraction of thiosulphate and free sulphides which can then be pumped back into the mantle cavity to their symbiont (Dufour & Felbeck, 2003; Dando *et al.*,; Hakonen *et al.*, 2010). Preliminary research has demonstrated significant diel fluctuation in the ventilation activities of

thyasirid bivalves and the associated reduction/oxidation reactions in the surrounding sediment (Dufour, pers. comm.). This raises the intriguing possibility that thyasirid bivalves may not only be able to alter the provisioning of sulphides to their symbionts through the chemotactic targeting of sulphide patches with their pedal mining behaviour (Dufour & Felbeck, 2003), but also may be capable of varying the degree and extent of their ventilation behaviours in the interest of controlling sulphide supply to their chemosynthetic bacterial symbionts. The rapid increase in symbiont division rates in *T. flexuosa* and *T. cf. gouldi*, coupled with the eventual host mortality observed in *T. flexuosa* highlight the importance of these behaviours in maintaining a mutually beneficial, regulated chemosymbiosis in thyasirid bivalves.

Additionally, these findings underscore an inherent weakness in the facultative, poorly-regulated nutritional symbioses such as that presented for *T. flexuosa* and *T. cf. gouldi*. The long term evolutionary stability of such symbioses is predicated on the direct cooperation between partners (Sachs *et al.*, 2011), involving the regulation of symbiont population dynamics and the restriction of bacterial cells to specific host tissues adapted to harbour them (Neckelmann & Muscatine, 1983). The fact that the mutualistic nature of the symbiosis between *T. flexuosa* and its bacteria is highly conditional on such a delicate balance in the provisioning of reduced sulphur species to its bacterial symbionts highlights the vulnerability of this system. The consequent deleterious effects of sulphide de-regulation on these thyasirid hosts may explain the patchy distribution of chemosymbiosis within this family (Dufour, 2005), and the potential for rapid evolutionary reversion to an asymbiotic state (Batstone *et al.*, 2014). Highly conditional mutualistic outcomes in the absence of complex mechanisms of host-symbiont co-

regulation and cooperation are predicted to lead to the possibility of the collapse and disappearance of symbiotic associations (Bronstein, 1994; Sachs & Simms, 2006).

The evolution of more complex and obligate chemosymbioses in thyasirids would likely necessitate the evolution of more complex and direct mechanisms of control over symbiont life cycles. This could involve the incorporation of tightly regulated cell differentiation and apoptosis cycles, such as those observed in *Riftia pachyptila* (Pflugfelder *et al.*, 2009), or the evolution of cellular mechanisms allowing for the direct repression of bacterial symbionts (Caro *et al.*, 2007; Kuwahara *et al.*, 2007). A shift to less fragile mechanisms of host regulation over symbiont life cycles may be theorized to result in the appearance of features of complex and evolutionary stable symbioses such as the intracellular localisation of symbionts and nutrient translocation, both of which symbiotic thyasirids generally lack (Dufour, 2005).

## Conclusions

Symbiont division occurs under natural conditions in the thyasirid clams *T. gouldi* and *T. flexuosa*. This division of bacterial symbionts may assist in the replacement of cells lost in the process of endocytosis and lysosomal lysis involved in nutrient transfer between symbiont and host. Furthermore, the discovery of dividing bacterial symbionts in two thyasirid species is inconsistent with the lack or rarity of symbiont replication in other symbiotic bivalve families such as the Lucinidae, Vesicomysidae and Solemyidae. Symbiont division in the Thyasiridae may point to the hosts' inability to directly control

or repress bacterial reproduction in association with host tissues, a fact that further cements the relatively simplistic and less derived nature of thyasirid symbioses.

Exposure to thiosulphate, a metabolic substrate for the chemosynthetic pathways of thyasirid symbionts, results in the rapid expansion of bacterial symbiont populations. The frequency of bacterial replication may be dependent on the concentration of thiosulphate to which they are exposed, and continued exposure to elevated thiosulphate levels results in the unrestrained expansion of the bacterial symbionts, ultimately leading to the death of the host organisms themselves. Symbiotic associations in the Thyasiridae are hypothesized to be highly fragile, and the inability of the hosts to exert direct control over the life cycles of their bacterial symbionts may make this system prone to conflicts of interest and dissolution. These findings may help in explaining the patchy distribution of symbiosis within the Thyasiridae and the potential for rapid evolutionary reversion to an asymbiotic state. Furthermore, the deleterious consequences of the rapid expansion of the symbiont population of *T. flexuosa* indicates the importance of controlling bacterial sulphide exposure in thyasirid symbioses. Behavioural adaptations such as the chemotactic pedal mining involved in sediment sulphide localization and diel fluctuations in ventilation of these sub-surface burrows may allow the host control over the chemical composition of the fluid surrounding its bacterial symbionts.

Symbioses between thyasirid bivalves must be considered as highly suitable model systems for the exploration of bacterial life cycle regulation and the control of symbiont population dynamics. The findings of this study may have widespread implications for the many relatively simplistic and facultative symbiotic associations in nature, including the diverse chemosymbiotic associations in marine invertebrates but



also the complex and highly fraught relationships between vertebrate hosts and their intestinal microflora (Xu & Gordon, 2003). The stability of mutualistic outcomes in facultative symbioses may be highly fragile if changes in host behaviour or local environment result in new nutrient regimes for the symbiont. Under extreme circumstances, this may lead to rapid de-regulation of the symbiont population and to the accumulation of severely deleterious consequences to the host or the evolutionary collapse of fraught and highly delicate facultative symbioses.

## Works Cited

- Allen, J. (1958). On the basic form and adaptations to habitat in the Lucinacea (Eulamellibranchia). *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences*. 241, 421-484.
- Arp, A. J., Childress J. J., & Fisher, C. R. (1984). Metabolic and blood gas transport characteristics of the hydrothermal vent bivalve *Calyptogena magnifica*. *Physiological Zoology*. 57:6, 648-662.
- Batstone, R.T., Laurich, J. R., Salvo, F., & Dufour, S. C. (2014). Divergent chemosymbiosis-related characters in *Thyasira* cf. *gouldi* (Bivalvia: Thyasiridae). *PLoS ONE*. 9:3, e92856.
- Bright, M., & Sorgo, A. (2003). Ultrastructural reinvestigation of the trophosome in adults of *Riftia pachyptila* (Annelida, Siboglinidae). *Invertebrate Biology*. 122, 347-368.
- Brissac, T., Mercot, H., & Gros, O. (2011). *Lucinidae*/sulfur-oxidizing bacteria: ancestral heritage of opportunistic association? Further insights from the Bohol Sea (the Philippines). *FEMS Microbial Ecology*. 75, 63-76.
- Bronstein, J.L. (1994). Conditional outcomes in mutualistic interactions. *Trends in Ecology & Evolution*. 9:6, 214-217.
- Caro, A., Got, P., Bouvy M., Troussellier, M., & Gros, O. (2009). Effects of long-term starvation on a host bivalve (*Codakia orbicularis*, Lucinidae) and its symbiont population. *Applied and Environmental Microbiology*. 75:10, 3304-3313.

- Caro, A., Gros, O., Got, P., De Wit, R., & Troussellier, M. (2007). Characterization of the population of the sulfur-oxidizing symbiont of *Codakia orbicularis* (Bivalvia, Lucinidae) by single-cell analyses. *Applied and Environmental Microbiology*. 73:7, 2101-2109.
- Cary, S.C., & Giovanni, S.J. (1993). Transovarian inheritance of endosymbiotic bacteria in clams inhabiting deep-sea hydrothermal vents and cold seeps. *Proceedings of the National Academy of Sciences of the United States of America*. 90, 5695-5699.
- Chaston, J. & Goodrich-Blair, H. (2010). Common trends in mutualism revealed by model associations between invertebrates and bacteria. *FEMS Microbiology Reviews*. 34, 41-58.
- Childress, J. J., Fisher, C. R., Favuzzi, J. A., Arp, A. J., & Oros, D. R. (1993). The role of a zinc-based, serum-borne sulphide-binding component in the uptake and transport of dissolved sulphide by the chemoautotrophic symbiont-containing clam *Calyptogena elongata*. *Journal of Experimental Biology*. 179, 131-158.
- Dando, P. R., & Southward, A. J. (1986). Chemoautotrophy in bivalve molluscs of the genus *Thyasira*. *Journal of the Marine Biology Association of the United Kingdom*. 66, 915-929.
- Dando, P.R., Southward A. J., & Southward, E. C. (2004). Rates of sediment sulphide oxidation by the bivalve mollusc *Thyasira sarsi*. *Marine Ecology Progress Series*. 280, 181-187.
- Dando, P. R., & Spiro, B. (1993). Varying nutritional dependence of the thyasirid bivalves *Thyasira sarsi* and *T. equalis* on chemoautotrophic symbiotic bacteria, demonstrated by isotope ratios of tissue carbon and shell carbonate. *Marine Ecology Progress Series*. 92, 151-158.
- Distel, D. L., & Cavanaugh, C. M. (1994). Independent phylogenetic origins of methanotrophic and chemoautotrophic bacterial endosymbiosis in marine bivalves. *Journal of Bacteriology*. 176, 1932-1938.
- Donval, A., Le Pennec, M., Herry, A., & Diouris, M. (1989). Nutritional adaptations of littoral bivalve molluscs to reducing biotopes. *Reproduction, Genetics and Distributions of Marine Organisms. Proceedings of the 23<sup>rd</sup> European Marine Biology Symposium*. 373-378.
- Douglas, A. E. (2008). Conflict, cheats and the persistence of symbioses. *New Phytologist*. 177, 849-858.

- Dubilier, N, Bergin, C., & Lott, C. (2008). Symbiotic diversity in marine animals: the art of harnessing chemosynthesis. *Nature Reviews Microbiology*. 6:10, 725-740.
- Dufour, S. C. (2005). Gill anatomy and the evolution of symbiosis in the bivalve family Thyasiridae. *Biological Bulletin*. 208, 200-212.
- Dufour, S. C., & Felbeck, H. (2003). Sulphide mining by the superextensile foot of symbiotic thyasirid bivalves. *Nature*. 426:6962, 65-67.
- Dufour, S. C., & Felbeck, H. (2006). Symbiont abundance in thyasirids (Bivalvia) is related to particulate food and sulphide availability. *Marine Ecology Progress Series*. 320, 185-194.
- Dufour, S. C., Laurich, J. R., Batstone, R. T., McCuaig, B., Elliot, A., & Poduska, K. M. (2014). Magnetosome-containing bacteria living as symbionts of bivalves. *The ISME Journal*. doi:10.1038/ismej.2014.93.
- Duperron, S., Gaudron, S.M., Rodrigues C.F., Cunha M.R., Decker C, & Olu, K. (2012a). An overview of chemosynthetic symbioses in bivalves from the North Atlantic and Mediterranean Sea. *Biogeosciences Discussions*. 9, 16815-16875.
- Duperron, S., Rodrigues, C. F., Léger, N., Szafranski, L., Decker, G., Olu, K., & Gaudron, S. M. (2012b). Diversity of symbioses between chemosynthetic bacteria and metazoans at the Guinness cold seep site (Gulf of Guinea, West Africa). *Microbiologyopen*. 1, 467-480.
- Fisher, C. R., & Childress, J. J. (1986). Translocation of fixed carbon from symbiotic bacteria to host tissues in the gutless bivalve *Solemya reidi*. *Marine Biology*. 93, 59-68.
- Gilbert, D., & Pettigrew, G. (1993). Current-meter data from Bonne Bay, Newfoundland during the summer of 1991. *Canadian Report of Hydrography and Ocean Sciences*. 122:63.
- Green-García, A. M., & Engel, A. S. (2012). Bacterial diversity of siliciclastic sediments in a *Thalassia testudinum* meadow and the implications for *Luciniscia nassula* chemosymbiosis. *Estuarine, Coastal and Shelf Science*. 112, 153-161.
- Gros, O., Darasse, A., Durand, P., Frenkiel, L., & Moueza, M. (1996). Environmental transmission of a sulfur-oxidizing bacterial gill endosymbiont in the tropical lucinid bivalve *Codakia orbicularis*. *Applied and Environmental Microbiology*. 62, 2324-2330.
- Gros, O., Liberge, M., Heddi, A., Khatchadourian, C., & Felbeck, H. (2003). Detection of the free-living forms of sulfide-oxidizing gill endosymbionts in the lucinid habitat

- (*Thalassia testudinum* environment). *Applied and Environmental Microbiology*. 69:10, 6264-6267.
- Gros, O., Elisabeth, N. H., Gustave, S. D. D., Caro A., & Dubilier, N. (2012). Plasticity of symbiont acquisition throughout the life cycle of the shallow-water tropical lucinid *Codakia orbiculata* (Mollusca: Bivalvia). *Environmental Microbiology*. 14:6, 1584-1595.
- Hakonen, A., Hulth, S., & Dufour, S. (2010). Analytical performance during radiometric long-term imaging of pH in bioturbated sediments. *Talanta*. 81, 1393-1401.
- Herre, E. A., Knowlton, N., Mueller, U. G., & Rehner, S. A. (1999). The evolution of mutualisms: exploring the paths between conflict and cooperation. *Trends in Ecology & Evolution*. 14:2, 49-53.
- Hoegh-Guldberg, O., & Hinde, R. (1986). Studies on a nudibranch that contains zooxanthellae I. Photosynthesis, respiration and the translocation of newly fixed carbon by zooxanthellae in *Pteraeolidia ianthina*. *Proceedings of the Royal Society of London. Series B, Biological Sciences*. 228:1253, 493-509.
- Jean, F., & Hily, C. (1993). Quantitative sampling of soft-bottom macroepifauna for assessing the benthic system in the Bay of Brest (France). *Oceanologica Acta*. 17:3, 319-330.
- Kleiner, M., Peterson, J. M., & Dubilier, N. (2012). Convergent and divergent evolution of metabolism in sulfur-oxidizing symbionts and the role of horizontal gene transfer. *Current Opinion in Microbiology*. 15, 621-631.
- Krueger, D. M., Gustafson, R. G., & Cavanaugh, C. M. (1996). Vertical transmission of chemoautotrophic symbionts in the bivalve *Solemya velum* (Bivalvia: Protobranchia). *Biological Bulletin*. 190:2, 195-202.
- Kuwahara, H., Yoshida, T., Yoshihiro, T., Shimamura, S., Nishi, S., Harada, M., Matsuyama, K., Takishita, M., Kawato, M., Uematsu, K., Fujiwara, Y., Sato, T., Kato, C., Kitagawa, M., Kato, I., & Maruyama, T. (2007). Reduced genome of the thioautotrophic intracellular symbiont in a deep-sea clam, *Calyptogena okutanii*. *Current Biology*. 17, 881-886.
- Law, R., & Lewis, D.H. (1983). Biotic environments and the maintenance of sex—some evidence from mutualistic symbioses. *Biological Journal of the Linnean Society*. 20:3, 249-276.
- Le Pennec, M., Diouris, M., & Herry, A. (1988a). Endocytosis and lysis of bacteria in gill epithelium of *Bathymodiolus thermophilus*, *Thyasira flexuosa*, and *Lucinella divaricata* (Bivalve, Molluscs). *Journal of Shellfish Research*. 7:3, 483-489.

- Le Pennec, M., Herry, A., Diouris, M., Moraga, D., & Donval, A. (1988b). Chimioautotrophie chez les Lucinacea, bivalves littoraux de milieux reducteurs. II- Caracteristiques morphologiques des bacteries symbiotiques et modifications structurelles adaptives des branchies de l'hote. *Haliotis*. 18, 207-217.
- Liberge, M., Gros, O., & Frenkiel, L. (2001). Lysosomes and sulfide-oxidizing bodies in the bacteriocytes of *Lucina pectinata*, a cytochemical and microanalysis approach. *Marine Biology*. 139, 401-409.
- Muscatine, L., Falkowski, P. G., Dubinsky, Z., Cook, P. A., & McCloskey, L. R. (1989). The effect of external nutrient resources on the population dynamics of zooxanthellae in a reef coral. *Proceedings of the Royal Society of London. Series B, Biological Sciences*. 236, 311-324.
- Muscatine, L., & Neckelmann, N. (1981). Regulation of numbers of algae in the *Hydra-Chlorella* symbiosis. *Berichte der Deutschen Botanischen Gesellschaft*. 94:1, 571-582.
- Nyholm, S. V., Song, P., Dang, J., Bruce, C., & Girguis, P. R. (2012). Expression and putative function of innate immunity genes under *in situ* conditions in the symbiotic hydrothermal vent tubeworm *Ridgeia piscesae*. *PLoS One*. 7, e38267.
- Neckelmann, N., & Muscatine, L. (1983). Regulatory mechanisms maintaining the *Hydra-Chlorella* symbiosis. *Proceedings of the Royal Society of London. Series B, Biological Sciences*. 219, 193-210.
- Nussbaumer, A. D., Fisher, C. R., & Bright, M. (2006). Horizontal endosymbiont transmission in hydrothermal vent tubeworms. *Nature*. 441, 35-348.
- Oliver, P. G., & Taylor, J. D. (2012). Bacterial symbiosis in the Nucinelidae (Bivalvia: Solemyida) with descriptions of two new species. *Journal of Molluscan Studies*. 78, 81-91.
- Oliver, P. G., Southward, E. C., & Dando, P. R. (2013). Bacterial symbiosis in *Syssitomya pourtalesiana* (Galeommatoidea: Montacutidae), a bivalve commensal with the deep-sea echinoid *Pourtalesia*. *Journal of Molluscan Studies*. 79, 30-41.
- Pflugfelder, B., Cary, S. C., & Bright, M. (2009). Dynamics of cell proliferation and apoptosis reflect different life strategies in hydrothermal vent and cold seep vestimentiferan tubeworms. *Cell and Tissue Research*. 337, 149-165.
- Rodrigues, C. F., & Duperron, S. (2011). Distinct symbiont lineages in three thyasirid species (Bivalvia: Thyasiridae) from the eastern Atlantic and Mediterranean Sea. *Naturwissenschaften*. 98:4, 281-287.

- Roeselers, G., & Newton, I. L. G. (2012). On the evolutionary ecology of symbioses between chemosynthetic bacteria and bivalves. *Applied Microbiology and Biotechnology*. 94, 1-10.
- Sachs, J. L., Essenberg, C. J., & Turcotte, M. M. (2011). New paradigms for the evolution of beneficial interactions. *Trends in Ecology & Evolution*. 26:4, 202-209.
- Sachs, J. L., & Simms, E. L. (2006). Pathways to mutualism breakdown. *Trends in Ecology & Evolution*. 21:10, 585-592.
- Southward, E. C. (1986). Gill symbionts in Thyasirids and other bivalve molluscs. *Journal of the Marine Biology Association of the United Kingdom*. 66, 889-914.
- Spiro, B., Greenwood, P. B., Southward, A. J., & Dando, P. R. (1986).  $^{13}\text{C}/^{12}\text{C}$  ratios in marine invertebrates from reducing sediments: confirmation of nutritional importance of chemoautotrophic endosymbiotic bacteria. *Marine Ecology Progress Series*. 28, 233-240.
- Stewart, F.J., & Cavanaugh, C.M. (2006). Bacterial endosymbioses in *Solemya* (Mollusca: Bivalvia)-model systems for studies of symbiont-host adaptation. *Antonie van Leeuwenhoek*. 90,343-360.
- Thorsen, B. K., Enger, O., Norland, S., & Hoff, K. J. (1992). Long-term starvation survival of *Yersinia ruckeri* at different salinities studied by microscopical and flow cytometry methods. *Applied and Environmental Microbiology*. 62, 4587-4593.
- Veiga, H., & Pinho, M. G. (2012). Bacterial cell division: what it takes to divide a prokaryotic cell. *Canal BQ*. 9, 18-26.
- Weiss, D. S. (2004). Bacterial cell division and the septal ring. *Molecular Microbiology*. 54(3): 588-597.
- Xu, J., & Gordon, J. I. (2003). Inaugral article: honor thy symbionts. *Proceedings of the National Academy of Sciences of the United States of America*. 100, 10452-10459.

## Thesis Summary

### Summary of results

This study sought out to address questions pertaining to the dynamic nature of mutualistic associations between sulphur-oxidizing bacterial symbionts and their thyasirid hosts. In Chapter 1, I examined seasonal variation in the relative symbiont abundance in *Thyasira gouldi* populations in Bonne Bay, NL. We tested the following null hypothesis by quantifying and analysing symbiont populations associated with the bacteriocytes of *T. cf. gouldi* over the 2011 and 2012 sampling seasons:

- (1) Symbiont abundance in *Thyasira cf. gouldi* is constant throughout the year; there is no seasonal effect on symbiont population size.

The results of this analysis conclusively disproved our null hypothesis, and there is indeed a strong component of seasonal variation affecting symbiont abundance in *T. cf. gouldi*. Symbiont density is greatest in the autumn, followed by a subsequent decline in bacterial populations towards late winter and early spring. Additionally, symbiont abundance was shown to correlate with clam shell length, indicating a probable effect of symbiont abundance on host fitness. This result is not surprising, as symbiont abundance was positively correlated with the density of membrane whorls, structures associated with the hosts' digestion of bacterial symbionts. As such, we can state conclusively that, in accordance with previously documented evidence of temporal variability in thyasirid symbiont abundance (Donval *et al.*, 1989; Dando & Spiro, 1993; Dufour & Felbeck, 2006), *T. cf. gouldi* displays prominent seasonal trends in symbiont abundance, and likely in the nutritional contribution of symbiotically derived carbon.

In Chapter 2, I examined the effect of media enrichment with reduced sulphur compounds on the bacterial symbiont populations in two thyasirids, *T. cf. gouldi* and *T. flexuosa*. In accordance with previous indications that the sulphur oxidizing bacterial symbionts of chemosymbiotic bivalves rarely, if ever, undergo division in association with host cells (Stewart & Cavanaugh, 2006; Caro *et al.*, 2007; Gros *et al.*, 2012), we formulated the following null hypothesis:

- (2) Bacterial symbionts do not undergo division in association with host gill epithelial cells; hosts can control bacterial population dynamics, and addition of reduced sulphur compounds does not result in an increase in bacterial division or population size.

Once again, the results of our experiment conclusively discredited our null hypothesis. Symbiont division was seen in the bacteriocytes of both *T. cf. gouldi* and *T. flexuosa* under natural conditions; that is, without the addition of reduced sulphur compounds. Enrichment of experimental media with dissolved thiosulphate resulted in a marked increase in the frequency of dividing bacterial symbionts, and in the case of *T. flexuosa*, resulted in the overwhelming of host gill epithelial cells via unrestrained bacterial division, leading to the death of host bacteriocytes and eventual mortality. These results illustrate that bacterial division occurs naturally in the symbionts of thyasirid bivalves, and symbiont populations are likely held in check by host behavioural adaptations regarding the supply of reduced sulphur compounds to their symbiont populations.



## Conclusions

Intrafamilial variation in the possession of sulphur-oxidizing bacterial symbionts is well documented in the Thyasiridae. Within this bivalve family, not all species possess bacterial symbionts, and this phylogenetic patchiness of symbiosis with chemoautotrophic bacteria can occur between closely related species (Dando & Southward, 1986; Dufour, 2005; Batstone *et al.*, 2014). Though well documented, the evolutionary forces responsible for producing this remarkable degree of variation in the possession of bacterial symbionts remain relative mysteries. Within the *Thyasira* cf. *gouldi* cryptic species complex that typifies the most extreme example of variation in the possession of bacterial symbionts (Batstone *et al.*, 2014), we have demonstrated conclusive evidence of strong, seasonal variability in symbiont abundance (Chapter 1). Relative symbiont abundances fluctuate greatly over the course of annual cycles, with bacterial abundance peaking in late autumn and reaching its minimum in late winter and early spring. This cycle is likely driven by temporal and spatial variability in the influx of organic matter to the benthos, primarily through spring and fall phytoplankton blooms as well as through the input of terrestrial organic matter (Tian *et al.*, 2001). Periodicity in organic matter enrichment creates temporal variation in sediment sulphide availability (Mudryk *et al.*, 2000; Faulwetter *et al.*, 2013), which is potentially further exacerbated by thyasirids' potential to oxygenate sediments and deplete them of reduced sulphur compounds (Dando *et al.*, 2004; Dando & Spiro, 1993). As such, it is highly likely that symbiont abundance in thyasirids is related to sediment sulphide availability, which may be driven by a combination of environmental (phytoplankton blooms, plant detritus) and biological (thyasirid bioirrigation) factors. Furthermore, large bacterial symbiont populations in *T.*

cf. *gouldi* appear to be positively correlated with host fitness, illustrating the potential for symbiotic nutritional input to constitute a strong selective force in symbiotic thyasirids. This highly labile scenario provides insight into the potential for the loss of symbiosis in the Thyasiridae (Batstone *et al.*, 2014) as well as the marked degree of intrafamilial variation in the possession of bacterial symbionts (Dufour, 2005); long term shifts in the environmental availability of reduced sulphur compounds may constitute a particularly strong driving force in the evolution and loss of chemosymbioses in the Thyasiridae.

Though the possession of large bacterial symbiont populations likely confers upon its host a notable fitness benefit, the association between thyasirids and their sulphur-oxidizing bacterial symbionts remains facultative, with the digestion of bacterial symbionts constituting an (albeit important) supplement to heterotrophic feeding (Dufour, 2005). In accordance with current theories on host symbiont co-evolution and method of symbiont transfer, the facultative symbionts of thyasirids are environmentally acquired, and maintain many genetic features of free living chemolithoautotrophic bacteria, such as flagella and magnetosome chains (Dufour *et al.*, 2014; B. McCuaig, pers. comm.). The diversity of bacteria forming symbiotic association with thyasirids (Rodrigues & Duperron, 2011) as well as their facultative nature and opportunistic acquisition (Dufour *et al.*, 2014), underscores the relative lack of specificity in thyasirid chemosymbiosis and their status as representatives of an early stage in the evolution of complex chemosymbioses (Roeselers & Newton, 2012). As such, it is not surprising that thyasirids lack the complex mechanisms of symbiont population control seen in other chemosymbiotic invertebrates such as *Riftia pachyptila* (Pflugfelder *et al.*, 2009), lucinid bivalves (Caro *et al.*, 2007; Caro *et al.*, 2009) and vesicomyid bivalves (Kuwahara *et al.*,

2007). Indeed, thyasirids are likely incapable of directly restraining bacterial population expansion in their gill filaments, and must rely on behavioural adaptations for the acquisition and supply of reduced sulphur species. The fact that thiosulphate enrichment resulted in the acceleration of bacterial symbiont division and the potential overgrowth of host tissues (Chapter 2) exposes an inherent weakness in the facultative, relatively less derived chemosymbiotic relationships of thyasirid bivalves. In this group, mutualistic outcomes are highly conditional, with the potential for bacterial exploitation of host tissues and subsequent deleterious consequences for the host organism. This creates a strong evolutionary tension between host and symbiont, introducing the possibility for the evolutionary loss of these mutualistic associations (Sachs & Simms, 2006). These findings may further explain the phylogenetic patchiness of chemosymbiosis and the potential for reversion to autonomy in the Thyasiridae, and also indicate that in the early, highly dynamic stages of the evolution of complex chemosymbioses, behavioural mechanisms such as pedal tract construction and burrow ventilation patterns (Dufour & Felbeck, 2003; Dando *et al.*, 2004) may play an important role in buffering mutualisms, ensuring positive outcomes and continued mutualistic interactions between partners in the absence of complex cellular, genetic, or biochemical mechanisms of controlling and regulating interactions between symbiont and host.

## Future directions

Though seasonal variation in symbiont abundance has been conclusively demonstrated in the *T. cf. gouldi* populations of the relatively pristine Bonne Bay, NL, it would be instructive to examine seasonal fluctuation in symbiont abundance in thyasirids inhabiting regions where organic matter enrichment is either less variable (eg. hydrothermal vents, cold seeps) or driven by non-cyclical factors (eg. sewage discharge). In conjunction with a study evaluating seasonal variation in thyasirids alongside measurements of sediment sulphide concentrations, this would allow us to conclusively determine whether or not temporal (potentially long and short term) variation in thyasirid symbiont abundance is driven by sulphide availability. Additionally, the effect of symbiont abundance on host reproductive fitness merits significant further investigation. The reproductive fitness of the symbiotic and asymbiotic *T. cf. gouldi* OTUs in Bonne Bay, NL (Batstone *et al.*, 2014) could be compared, whilst altering the chemical composition of sediments and the supply of particulate organic matter to symbiotic thyasirids could yield important insights into the relationship between symbiont population size and host fitness.

The presence of dividing bacterial symbionts should be investigated in other thyasirid species, as a general consensus on the mechanism of symbiont population renewal is currently lacking in the thyasirid literature. A more refined study investigating the effect of thiosulphate enrichment on chemosymbiotic bivalves, including both thyasirids and other facultative chemosymbiotic organisms such as the Lucinidae, would similarly yield fascinating results regarding different mechanisms of symbiont population control in chemosymbiotic organisms. The inclusion of more concentrated thiosulphate

treatments in thyasirid species other than *T. flexuosa* would allow us to confirm that unrestrained bacterial division leads to host mortality, and identify the threshold thiosulphate concentrations beyond which hosts lose the ability to respond to bacterial population expansion through the digestion of bacterial symbionts. Finally, since it is now apparent that host behavioural mechanisms relating to the construction of pedal tracts, bioirrigation and sulphide acquisition are crucial in maintaining stable bacterial symbiont populations, further research should investigate the method of sulphide acquisition in thyasirids, along with potential diel fluctuations in ventilation patterns, which may allow thyasirids to regulate symbiont thiosulphate exposure by alternating the uptake of sulphidic porewater with the intake of oxygenated surface water.

## Works Cited

- Batstone, R. T., Laurich, J. R., Salvo, F., & Dufour, S. C. (2014). Divergent chemosymbiosis-related characters in *Thyasira* cf. *gouldi* (Bivalvia: Thyasiridae). *PLoS ONE*.9:3, e92856.
- Caro, A., Gros, O., Got, P., De Wit, R., & Troussellier, M. (2007). Characterization of the population of the sulfur-oxidizing symbiont of *Codakia orbicularis* (Bivalvia, Lucinidae) by single-cell analyses. *Applied and Environmental Microbiology*. 73:7, 2101-2109.
- Caro, A., Got, P., Bouvy, M., Troussellier, M., & Gros, O. (2009). Effects of long-term starvation on a host bivalve (*Codakia orbicularis*, Lucinidae) and its symbiont population. *Applied and Environmental Microbiology*. 75:10, 3304-3313.
- Dando, P. R., & Southward, A. J. (1986). Chemoautotrophy in bivalve molluscs of the genus *Thyasira*. *Journal of the Marine Biology Association of the United Kingdom*. 66, 915-929.
- Dando, P.R., Southward A. J., & Southward, E.C. (2004). Rates of sediment sulphide oxidation by the bivalve mollusc *Thyasira sarsi*. *Marine Ecology Progress Series*. 280, 181-187.

- Dando, P. R., & Spiro, B. (1993). Varying nutritional dependence of the thyasirid bivalves *Thyasira sarsi* and *T. equalis* on chemoautotrophic symbiotic bacteria, demonstrated by isotope ratios of tissue carbon and shell carbonate. *Marine Ecology Progress Series*. 92, 151-158.
- Donval, A., Le Pennec, M., Herry, A., & Diouris, M. (1989). Nutritional adaptations of littoral bivalve molluscs to reducing biotopes. In J. S. Ryland, & P. A. Tyler (Eds.), *Reproduction, Genetics and Distributions of Marine Organisms. Proceedings of the 23rd European Marine Biology Symposium*. 373-378. Swansea.
- Dufour, S. C. (2005). Gill anatomy and the evolution of symbiosis in the bivalve family Thyasiridae. *Biological Bulletin*. 208:3, 200-212.
- Dufour, S.C., & Felbeck, H. (2003). Sulphide mining by the superextensile foot of symbiotic thyasirid bivalves. *Nature*. 426:6962, 65-67.
- Dufour, S. C., & Felbeck, H. (2006). Symbiont abundance in thyasirids (Bivalvia) is related to particulate food and sulphide availability. *Marine Ecology Progress Series*. 320, 185-194.
- Dufour, S. C., Laurich, J. R., Batstone, R. T., McCuaig, B., Elliot, A., & Poduska, K. M. (2014). Magnetosome-containing bacteria living as symbionts of bivalves. *The ISME Journal*. doi:10.1038/ismej.2014.93.
- Faulwetter, J.L., Burr, M.D., Parker, A.E., Stein, O. R., & Camper, A.K. (2013). Influence of season and plant species on the abundance and diversity of sulfate reducing bacteria and ammonia oxidizing bacteria in constructed wetland microcosms. *Microbial Ecology*. 65:1, 11-127.
- Gros, O., Elisabeth, N.H., Gustave, S.D.D., Caro A., & Dubilier, N. (2012). Plasticity of symbiont acquisition throughout the life cycle of the shallow-water tropical lucinid *Codakia orbiculata* (Mollusca: Bivalvia). *Environmental Microbiology*. 14:6, 1584-1595.
- Kuwahara, H., Yoshida, T., Yoshihiro, T., Shimamura, S., Nishi, S., Harada, M., Matsuyama, K., Takishita, M., Kawato, M., Uematsu, K., Fujiwara, Y., Sato, T., Kato, C., Kitagawa, M., Kato, I., & Maruyama, T. (2007). Reduced genome of the thioautotrophic intracellular symbiont in a deep-sea clam, *Calyptogena okutanii*. *Current Biology*. 17, 881-886.
- Mudryk, Z., Podgórska, B., Ameryk, A., & Bolałek, J. (2000). The occurrence and activity of sulphate - reducing bacteria in the bottom sediments of the Gulf of Gdańsk. *Oceanologia Acta*. 42, 105-117.

- Pflugfelder, B., Cary, S. C., & Bright, M. (2009). Dynamics of cell proliferation and apoptosis reflect different life strategies in hydrothermal vent and cold seep vestimentiferan tubeworms. *Cell and Tissue Research*. 337, 149-165.
- Rodrigues, C. F., & Duperron, S. (2011). Distinct symbiont lineages in three thyasirid species (Bivalvia: Thyasiridae) from the eastern Atlantic and Mediterranean Sea. *Naturwissenschaften*. 98:4, 281-287.
- Roeselers, G., & Newton, I. L. G. (2012). On the evolutionary ecology of symbioses between chemosynthetic bacteria and bivalves. *Applied Microbiology and Biotechnology*. 94:1, 1-10.
- Sachs, J. L., & Simms, E.L. (2006). Pathways to mutualism breakdown. *Trends in Ecology & Evolution*. 21:10, 585-592.
- Stewart, F. J., & Cavanaugh, C. M. (2006). Bacterial endosymbioses in *Solemya* (Mollusca: Bivalvia)- model systems for studies of symbiont-host adaptation. *Antonie Van Leeuwenhoek*. 90:4, 343-360.
- Tian, R.C., Vezina, A. F., Starr, M., & Saucier, F. (2001). Seasonal dynamics of coastal ecosystems and export production at high latitudes: a modeling study. *Limnology and Oceanography*. 46:8, 1845-1859.

## Appendices

**Appendix 1 (next page):** Batstone, R. T., Laurich, J. R., Salvo, F., & Dufour, S. C. (2014). Divergent chemosymbiosis-related characters in *Thyasira* cf. *gouldi* (Bivalvia: Thyasiridae). *PLoS ONE*.9:3, e92856.



# Divergent Chemosymbiosis-Related Characters in *Thyasira* cf. *gouldi* (Bivalvia: Thyasiridae)

Rebecca T. Batstone, Jason R. Laurich, Flora Salvo, Suzanne C. Dufour\*

Department of Biology, Memorial University of Newfoundland, St. John's, Newfoundland, Canada

## Abstract

Within the marine bivalve family Thyasiridae, some species have bacterial chemosymbionts associated with gill epithelial cells while other species are asymbiotic. Although the abundance of symbionts in a particular thyasirid species may vary, the structure of their gills (i.e., their frontal-abfrontal thickening) does not. We examined gill structure in a species tentatively identified as *Thyasira gouldi* from a Northwest Atlantic fjord (Bonne Bay, Newfoundland) and found remarkable differences among specimens. Some individuals had thickened gill filaments with abundant symbionts, while others had thin filaments and lacked symbionts. We could differentiate symbiotic and asymbiotic specimens based on the size and outline of their shell as well as 18S rRNA, 28S rRNA and CO1 sequences. The wide morphological, genetic and symbiosis-related disparity described herein suggests that chemosymbiosis may influence host divergence, and that *Thyasira gouldi* forms a cryptic species complex.

**Citation:** Batstone RT, Laurich JR, Salvo F, Dufour SC (2014) Divergent Chemosymbiosis-Related Characters in *Thyasira* cf. *gouldi* (Bivalvia: Thyasiridae). PLoS ONE 9(3): e92856. doi:10.1371/journal.pone.0092856

**Editor:** Matthias Horn, University of Vienna, Austria

**Received:** September 25, 2013; **Accepted:** February 27, 2014; **Published:** March 21, 2014

**Copyright:** © 2014 Batstone et al. This is an open-access article distributed under the terms of the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited.

**Funding:** This project was funded by the Natural Sciences and Engineering Research Council of Canada (Discovery Grant: SCD and Undergraduate Student Research Award: JRL), and Newfoundland and Labrador Research and Development Corporation (Ignite R&D Grant: SCD and Ocean Industries Student Research Award: RTB). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Competing Interests:** The authors have declared that no competing interests exist.

\* E-mail: sdufour@mun.ca

## Introduction

The Thyasiridae (superfamily Thyasiroidea [1]) is one of five bivalve families that have established symbiotic relationships with chemosymbiotic bacteria [2]. Not all species within the family are chemosymbiotic [3,4], and among symbiotic species, nutritional reliance on symbionts varies [5]. In all but one chemosymbiotic thyasirid species, the bacterial symbionts are extracellular [3,6], residing either in enlarged spaces limited by the microvilli and the cell membrane in the bacteriocyte zone of modified, abfrontally expanded gill filaments, or among the microvilli of abfrontal epithelial cells in gills with shorter filaments [4]. Ultrastructural evidence indicates that thyasirid gills periodically engulf and digest their symbionts [3,5,7,8], and there is no evidence for the direct transfer ("milking") of nutrients from extracellular symbionts to thyasirids. Relationships between thyasirids and their extracellular symbionts may be relatively less specific or complex than in other groups where symbionts are intracellular.

Symbiont presence in the Thyasiridae has been linked to gill structure [4], and a recent molecular phylogeny suggests that symbiotic species belong to more than one distinct clades within the family [1]. One well-supported clade consists of *Thyasira flexuosa* Montagu, 1803, *T. gouldi* Philippi, 1845 and *T. polygona* Jeffreys, 1864, species with abfrontally thickened gills typically containing large numbers of symbionts [3,4,9]. Both *T. flexuosa* and *T. gouldi* reportedly have large geographic ranges [10,11], and specimens examined from various sites show similar features and abundant symbionts [3,4]. Although symbiont abundance can change according to particulate food availability [9], the structure of chemosymbiotic bivalve gills, i.e., the degree of frontal-abfrontal

elongation of filaments, or 'gill type' [4], has not been shown to vary along with symbiont abundance within a host species.

*Thyasira gouldi* is considered to be a panarctic species, usually found in organically enriched clay-grade sediments at < 50 m depth [11–13]. *T. gouldi* was initially collected in deep water off Massachusetts [14,15], and has been sampled from various cold water estuarine and fjord sites, including Scottish Sea Lochs, Southern Norwegian fjords, and the Southwest coast of the United Kingdom [11,16]. We recently sampled thyasirids, tentatively identified as *T. gouldi* (hereafter referred to as *T. cf. gouldi*) from a fjord in Bonne Bay, Newfoundland, Canada. The Bonne Bay specimens share shell characteristics with *T. gouldi* described from the eastern and western Atlantic [11,15], and resemble described *T. gouldi* specimens from the eastern Atlantic in main features of their internal anatomy [11] – we are unaware of descriptions of the internal anatomy for western Atlantic specimens.

Here, we report striking differences in symbiont presence and gill filament morphology among *Thyasira* cf. *gouldi* specimens from Bonne Bay, where many individuals contain thickened gills with abundant symbionts while others have thin gill filaments that lack symbionts. We hypothesize that symbiotic and asymbiotic specimens also differ in other characters (size, internal anatomy, shell shape, prodissoconch size, and partial 18S rRNA, 28S rRNA and CO1 gene sequences), and form more than one morphologically and genetically distinct groups.

## Materials and Methods

### Thyasirid sampling

Permits to collect invertebrates from Bonne Bay for experimental purposes were obtained from Fisheries and Oceans Canada.

Thyasirids were sampled from Bonne Bay (49°30'N 57°55'W), a fjord partially separated from the Gulf of St. Lawrence by a 50 m deep sill retaining a deep layer of cold water year round [17]. Using a Peterson grab, we collected specimens from three sites within East Arm: Southeast Arm (S, 49°27'51.46"N, 57°43'09.04"W, 30 m depth), Deer Arm (D, 49°32'43.48"N, 57°50'28.45"W, 30 m depth), and Neddy's Harbour (N, 49°31'21.44"N, 57°52'11.07"W, 15 m depth) in May and August 2010, and April, June, October, and December 2011. Sediments were sieved (1 mm mesh) to retrieve thyasirids, and 152 specimens are analysed herein. We carefully removed the gills from each specimen, keeping one gill for morphological analyses (N = 124), molecular analysis (N = 104), or another purpose. We obtained overlapping morphological and molecular data for 76 of the 152 specimens.

The shell length (anterior-posterior) was recorded for all specimens. We compared the shell length of symbiotic and asymbiotic thyasirids (following morphological and molecular confirmation; N = 76) using LS analysis in JMP; no major violations of assumptions were found based on the diagnostic residual plots observed. The valves of a subset of individuals (N = 37) were retained for shell shape analysis and prodissococonch measurements.

#### Light and Transmission Electron Microscopy of Gills

Gills retained for morphological analysis (N = 124) were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer for 24 hours, post-fixed in 1% osmium tetroxide in the same buffer for 1 h, dehydrated in an ascending ethanol series, and embedded in EPON resin. Semi-thin (1 µm) sections were made using a LKG Bromma 8800 ultramicrotome, and stained with 1% toluidine blue in 1% sodium borate for light microscopy. Ultra-thin sections (60 nm) of each gill were post-stained with uranyl acetate and lead citrate and observed using a Philips 300 transmission electron microscope.

#### Shell Shape Analysis and Prodissococonch Measurement

To test for differences in shell outline among specimens, we performed statistical analyses of Elliptic Fourier coefficients, landmark and size independent descriptors of shapes that are particularly suited for comparisons of bivalve shells [18]. The left valve of 37 individuals was imaged using a dissecting microscope. Outlines of each shell were digitized using the tool E-Snake [19] in ImageJ [20] and analyzed using SHAPE 1.3a [21]. Each shell outline was normalized for size and rotated using the umbo as reference, and Elliptic Fourier coefficients (EFCs) were then determined for each shell. A Principal Components Analysis (PCA) of the variance-covariance matrix was run to summarize the shape variation based on EFCs [18], and a graph produced to show the distribution of individual shell shapes along the first two principal components. Reconstructions of the shell contours at each extremity of the first two significant axes of the PCA were then made. PCA matrix coordinates (from significant axes) were used to compare the different shells in a cluster analysis in Primer 6.0 [22] based on Euclidean distance. A first cluster analysis was run based on the 10 first harmonics to discern individuals based on general differences in shape, and a t-test in Statistica was used to compare shell lengths between isolated groups. A second PCA and cluster analysis was performed based on one of the groups of shells identified by the first cluster analysis, using the 20 first harmonics (without the first) to decrease the importance of the general outline of the shell (described by the first harmonic) and therefore emphasize finer differences in outline [18]. An ANOSIM (999

permutations) was performed in Primer 6.0 on the second cluster to investigate OTU groupings.

We imaged the umbonal region of the valves retained for shape analysis and measured prodissococonch length (to 0.1 mm accuracy) using a measuring tool in ImageJ [20], and used a t-test in Statistica to compare the prodissococonch length of symbiotic and asymbiotic individuals. Because the prodissococonch of some specimens was damaged, we report measurements from 25 of those 37 valves.

#### DNA extraction, PCR amplification and sequencing

Considering that there are available 18S and 28S rRNA gene sequences of *Thyasira gouldi* from Mill Bay, Salcombe, UK [23] and Firth of Forth, UK [24], we chose to sequence this gene in the Bonne Bay specimens. These nuclear genes are useful in phylogenetic studies at various systematic scales because they contain both variable and highly conserved regions [25]. In addition, we also sequenced the more rapidly evolving CO1 gene, often used for species barcoding, although there are no available *T. gouldi* CO1 sequences in GenBank.

Gills (N = 104) were individually kept in 95% ethanol upon dissection. We isolated and purified DNA using the QIAgen DNeasy Blood and Tissue kit, following the spin-column protocol for animal tissues.

Polymerase chain reactions (PCRs) were performed using gene-specific sets of primers: 1) 18S-5' [26] and 18S1100R [27] for a ~1000 base pair (bp) fragment of the 18S rRNA gene; 2) LSU-5' [28] and LSU1600R [27] for a ~1500 bp fragment of the 28S rRNA gene; and 3) BivF4\_t1 and BivR1\_t1 [29] for a 667 bp fragment of the CO1 gene. For the nuclear genes, we performed 25 µL PCR using the Promega PCR Master Mix (Promega Corp.) containing 1 µL of template DNA, 50 µL/mL Taq DNA polymerase, 400 µM of each dNTP, 3.0 and 2.5 mM of MgCl<sub>2</sub> for 18S and 28S, respectively, and reaction buffer at a pH of 8.5. Thermocycling was run as follows: 4 min of initial denaturation at 94°C, followed by 35 cycles at 94°C for 30 s, 30 s at annealing temperatures of 54°C and 52°C for 18S and 28S, respectively, 2 min at 72°C, with a final elongation at 72°C for 5 min. Products filtered using Acro-Pro 100K-Omega filters (Pall Life Sciences) were prepared for sequencing using BigDye Terminator v 3.1 Cycle Sequencing Ready Reaction Mix (Applied Biosystems) and electrophoresed on an Applied Biosystems 3730 DNA Analyzer automated capillary sequencer. Amplification and sequencing of the CO1 gene was performed as in [29].

#### Sequence analyses

We aligned and compared forward and reverse sequences using Sequencher (v. 5.0, Gene Codes Corp.) and used Basic Local Alignment Search Tool (BLAST [30]) to find closely related sequences in GenBank. Operational taxonomic units (OTUs) were identified using MEGA5 [31] by aligning and then grouping individual 18S and 28S thyasirid sequences based on polymorphic nucleotide sites; sequences were therefore identical within OTUs (i.e., no base discrepancies excluding ambiguous sites). All alignments were executed with default values in MEGA5, using ClustalW [32]. We identified and removed poorly aligned sites using Gblocks v. 0.91b [33].

We determined the relatedness within and between our OTU pairs by calculating the average evolutionary divergence (*d*) for CO1 fragments by constructing a distance matrix using “*p*-distance” as the evolutionary model in MEGA5; thus, the proportion of base discrepancies could be compared between each pair of OTUs, accounting for fragment size. We used 2000 bootstrap replicates to generate standard error values for each



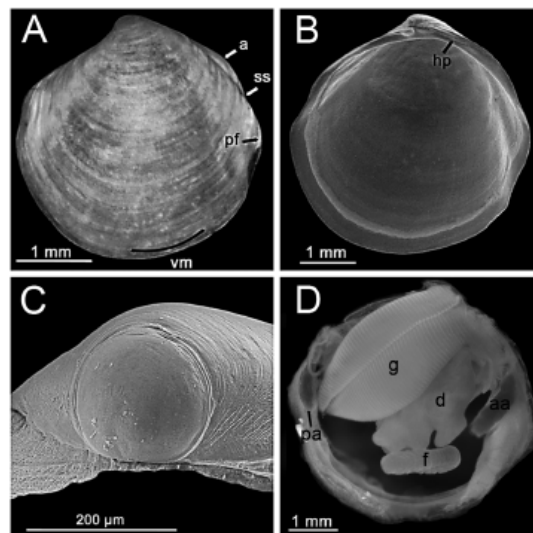
distance comparison, and treated gaps or missing data with pairwise deletion. To determine whether the calculated  $d$  differed within and between OTUs, we conducted a one-way ANOVA in RStudio (R v. 3.0.0). Tukey's HSD post-hoc tests were used to determine which group comparisons were significantly different.

## Results

### Anatomical characters and size

The shells of all specimens were generally equilateral-ovate, slightly higher than long, and bisinuate (Fig. 1a,b); shell dorso-ventral length and antero-ventral curvature slightly varied among specimens. A weakly projecting auricle defined the posterior region, with a submarginal sulcus forming a marginal sinus and a posterior fold forming a posterior sinus. The hinge plate lacked a clear cardinal tooth (Fig. 1b). Many individuals had patches of rust-coloured deposits on the anterior and posterior ends of their shells. Prodissoconch length ranged between 180 and 210  $\mu\text{m}$  (N=25; Fig. 1c).

In all specimens, gills had two demibranchs, the mantle margin was thickened at the anterior end, the foot was elongate and vermiform, and digestive diverticula formed a single mass (i.e., not as branched as in other thyasirid species) (Fig. 1d). The size range of all individuals we retrieved on the 1 mm mesh was between 1.8 – 5.0 mm.



**Figure 1. Morphology of *Thyasira cf. gouldi* from Bonne Bay, Newfoundland.** A. Outer view of the left shell valve, with a weakly projecting auricle (a), a well-defined submarginal sulcus (ss) forming a marginal sinus, a distinct, yet rounded, posterior fold (pf), and a rounded ventral margin (vm). B. Inner view of the right valve, revealing the absence of dentition along the hinge plate (hp). C. Scanning electron micrograph of the larval shell (~181  $\mu\text{m}$  diameter). D. Internal anatomy, showing a gill (g) with two demibranchs, the digestive diverticula (d), foot (f), anterior adductor (aa), posterior adductor (pa) and mantle margin, thickened at the anterior end.  
doi:10.1371/journal.pone.0092856.g001

### Gill morphology, symbiont presence, and corresponding characters

Out of the 76 specimens from which we had corresponding morphological and molecular data, we observed two dramatically different gill filament morphologies. Most specimens (N=55) had opaque, pink to white gills, with 'type 3' filaments as described in [4] (Fig. 2a). These gills were abfrontally expanded, with a clear bacteriocyte zone abfrontal to the frontal ciliated zone, and TEM observations revealed large numbers of extracellular symbionts (Fig. 2b). In contrast, 21 individuals had thin, translucent 'type 2' gills, conspicuously lacking a bacteriocyte zone (Fig. 2c). The abfrontal epithelium was pseudostratified, with apical cells (c2) overlying more basal cells (c1), the latter containing numerous enlarged mitochondria (Fig. 2d, e). Among those individuals with type 2 gills, we saw no symbiotic bacteria and very little toluidine blue staining (apart from mucocytes) in all sections observed.

In post-hoc analyses, we were unable to distinguish symbiotic and asymbiotic individuals based on the structure of any organ besides the gills, which generally appeared thicker and more opaque in symbiotic individuals. We noticed that the ferruginous patch on the posterior end of the shell was often located further dorsally in symbiotic individuals than in asymbiotic ones.

The shell size of symbiotic individuals ( $3.25 \pm 0.095$  mm) was significantly greater than that of asymbiotic individuals ( $2.50 \pm 0.077$  mm) [ $F(1, 75) = 3.97$ ,  $p = 1.25 \times 10^{-5}$ ]. However, the prodissoconch length of symbiotic (average  $\pm$  SD:  $200 \pm 23$   $\mu\text{m}$ , N=18) and asymbiotic (average  $\pm$  SD:  $193 \pm 9$   $\mu\text{m}$ , N=7) individuals did not differ significantly (t-test,  $p > 0.25$ ).

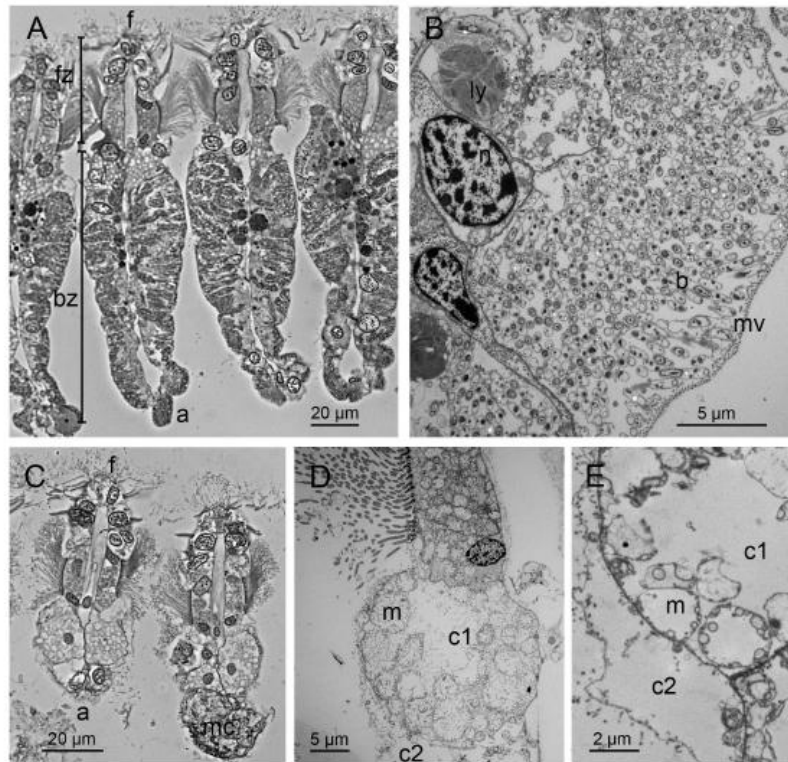
### Molecular descriptions: OTUs

18S fragments (size range: 960 – 1001 bp) were successfully sequenced from 104 individuals, all most closely matching *Thyasira gouldi* from Mill Bay, Salcombe, UK (GenBank accession number JF899224) with a sequence similarity of 99%. In 84 cases, corresponding 28S fragments (range: 1232 – 1448 bp) were successfully sequenced, all most closely matching *Thyasira gouldi* from Mill Bay, Salcombe, UK (JF899196) with a slightly lower sequence similarity (98%). We defined three distinct Operational Taxonomic Units (OTUs) in which 3 sites (out of 1001) and 13 sites (out of 1448) were polymorphic within the 18S and 28S fragments, respectively (Table 1). Individuals grouping together for the 18S fragment also grouped together for the 28S fragment. GenBank accession numbers are in Table 2.

Further confirmation of OTU groupings was obtained through mitochondrial CO1 sequences. We successfully obtained CO1 sequences (range: 256–667 bp) from 75 of the above-mentioned 104 individuals; closest matches in GenBank corresponded to *Thyasira obsoleta* (AM706507, 84% similarity) and *T. ferruginea* (AM706499, 83% similarity). Although the CO1 sequences were variable, there was strong support for three groups, corresponding exactly to OTUs 1, 2 and 3 defined by 18S & 28S sequences (100% posterior probability (PP) based on Bayesian reconstruction [data not shown]).

Based on the 76 specimens from which we had corresponding gill ultrastructure data, all individuals from OTUs 1 (N=49) and 2 (N=6) were symbiotic, while all individuals from OTU 3 (N=21) were asymbiotic.

The molecular divergence values within and between pairs of OTUs ( $d$ ), based on CO1, are in Table 3. The one-way ANOVA comparing  $d$  values within and between OTUs was statistically significant [ $F(2, 2340) = 115144$ ,  $p < 0.0001$ ]. We did not observe any major violations of ANOVA assumptions. The Tukey's HSD post-hoc test revealed significant differences when comparing within and between OTU  $d$  values, average within-OTU  $d$  being



**Figure 2. Light and electron micrographs of gill filaments of *Thyasira cf. gouldi*.** **A.** Symbiotic specimen. Light micrograph of a semi-thin, transverse section through four gill filaments. The ciliated frontal zone (fz) and bacteriocyte zone (bz) are highlighted. a: abfrontal end of a filament; f: frontal end of a filament. **B.** Symbiotic specimen. TEM of cells in the bacteriocyte zone of a gill filament, showing abundant bacteria (b), maintained extracellularly in pockets limited by extensions of host cell cytoplasm bearing microvilli (mv). Nuclei (n) and lysed remains of digested symbionts (ly) are visible in the host cell cytoplasm. **C.** Asymbiotic specimen. Light micrograph of a semi-thin, transverse section through two gill filaments. Note the shorter frontal (f) - abfrontal (a) length. mc: mucocyte. **D, E.** Asymbiotic specimen. TEM of cells in the abfrontal zone of a gill filament. Note the absence of bacteria. The epithelium is pseudostratified, with apical cells (c2) overlying basal cells (c1) containing large mitochondria (m). mv: microvilli.

doi:10.1371/journal.pone.0092856.g002

one order of magnitude lower (range: 0.00071 - 0.01116) than average between-OTU  $d$  (range: 0.1152 to 0.1178) (Table 3).

### Shell shape analysis

The first cluster analysis isolated two groups at a distance of 0.075 (Fig. 3). The first group (A) contained only symbiotic individuals (18 OTU 1) whereas the second group (B) included all three OTUs (5 OTU 1, 4 OTU 2, 10 OTU 3). Specimens from group A were larger than those from group B (t-test,  $p < 0.01$ , Fig. 3).

The first PCA, based on the 10 first harmonics (40 values per shell), provided 4 significant principal components (PC). PC1 represented at least 73% of the variability observed (Fig. 4), and two groups of individuals could be separated along this axis (corresponding to groups A and B in Fig. 3). Reconstruction of outlines at both extremities of this axis showed differences in the length/height ratio and anterior curvature, with specimens from group A characterized by a more equilateral ovate polygonal outline and specimens from group B being more subequilateral subovate (Fig. 4; terminology as in [13]). The second axis

explained only 8.7% of the variability, based upon the curvature of the anterior-ventral margin; symbiotic and asymbiotic specimens could not be discriminated along this axis.

A second analysis performed on specimens from group B (20 harmonics, with the first one removed) revealed 11 significant PCs. The cluster on the PCA values grouped most OTU 3 individuals together at a distance of 0.02 (Fig. 5); ANOSIM results indicated significant differences between OTU groups ( $R = 0.39$ ,  $p < 0.01$ ), with greater separation between OTUs 1 and 3 ( $R = 0.40$ ) and OTUs 2 and 3 ( $R = 0.52$ ) than between the symbiotic OTUs 1 and 2 ( $R = -0.04$ ). PC1 explained 23.97% of the observed variability, and outline reconstructions showed that symbiotic individuals tend to have a more sinuate posterior margin, a more pronounced anterior-ventral expansion, and a more sloping lunule margin than asymbiotic individuals, which were more rounded and had a more pronounced beak (Fig. 6). Along PC2 (18.95% of variability), symbiotic and asymbiotic individuals could not be separated.



**Table 1.** Polymorphic nucleotide sites within sequenced 18S and 28S gene fragments of *Thyasira cf. gouldi* OTUs.

OTU																
	Collection site	18S				28S										
		229	233	283	729	746	773	781	788	790	797	824	885	971	990	995
1 D(9), N(21), S(37)		C	C	T	C	C	T	G	C	T	A	C	T	C	C	T
2 D(3), N(5), S(1)		.	T	.	T	.	.	A	T	C	G	.	A	A	T	C
3 D(19), S(9)		T	T	A	T	T	C	A	.	.	.	A	A	A	T	C

The number of individuals of each OTU sequenced from each collection site (Deer Arm, D; Neddy's Harbour, N; Southeast Arm, S) is indicated in parentheses. Dots indicate the same base as in the top row.  
doi:10.1371/journal.pone.0092856.t001

### Geographic distribution

Within Bonne Bay, symbiotic individuals were found at all three sampling sites. Asymbiotic individuals were slightly more common than symbiotic individuals in Deer Arm, were relatively rare in Southeast Arm, and were absent altogether in Neddy's Harbour (Fig. 7).

### Discussion

#### Species identification

All specimens in this study could be assigned to the species *Thyasira gouldi* based on shell characteristics, including: 1) an equilateral-ovate outline, 2) a well-defined submarginal and posterior fold, 3) the presence of an auricle, and 4) a narrowly rounded ventral margin, appearing slightly angulate in several of the specimens [11]. As in other thyasirids [1], shell outline is thought to be highly variable in this species [11]; this was observed in our sampling. Moreover, the large prodissococonch (i.e., readily visible by eye) observed in the Bonne Bay specimens is a key characteristic of *Thyasira gouldi* [11,15]. This identification is further confirmed by 18S and 28S gene sequences, which are most similar to those of *Thyasira gouldi* from Mill Bay, Salcombe, UK. However, because of consistent differences in several of the studied characters, as detailed below, we remain uncertain about the species determination of Bonne Bay specimens and designate them as *Thyasira cf. gouldi* until further testing of species delineation hypotheses can be done.

#### Differences in symbiosis-related characters

Our examination of over 150 *T. cf. gouldi* specimens from Bonne Bay revealed a high degree of disparity in examined characters among specimens. Most remarkable was the finding of both symbiotic and asymbiotic individuals within this group, at each sampling date, and often within very close proximity (i.e., the same grab). To our knowledge, this is the first report of such a highly

divergent symbiotic condition among bivalves appearing to belong to a single species.

The differences observed did not only concern symbiont abundance, as previously documented in *Thyasira equalis* [4] and produced experimentally in *T. flexuosa* [9]. Rather, we observed striking and consistent differences in gill morphology (frontal-abfrontal thickness) that were not the result of differences in sectioning angle. In post-larval bivalves with homorhabdic filibranch gills, the frontal-abfrontal thickness of filaments remains relatively constant as gill filaments differentiate [34,35]. Our specimens had either gill type 2 or 3 [4], regardless of their size (> 1 mm shell length; we did not study smaller individuals). Gill type was also clearly associated with symbiont presence: there were no specimens with symbiotic type 2 gills, or asymbiotic type 3 gills. Those observations suggest that symbionts play important roles in modulating the development of this structure. In the symbiotic clam *Codakia orbicularis*, the frontal-abfrontal thickening of gill filaments (associated with the development of bacteriocytes) is triggered by exposure to their environmentally-transmitted symbionts during the juvenile stage [36]. Similarly, the early postembryonic development of the symbiotic organ of the squid *Euprymna scolopes* is controlled by bacterial symbionts [37], and rates of proliferation of zebrafish intestinal epithelial cells are regulated by the resident microbiota [38]. The possibility that thyasirid symbionts might similarly influence the development of gill filaments is intriguing and merits further study.

Symbiotic specimens attained a larger body size than asymbiotic individuals, probably as a result of greater trophic efficiency in the former group. The additional nutritional input gained from symbionts was hypothesized to be the cause of larger body size in symbiotic compared to asymbiotic juveniles of *Codakia orbicularis*

**Table 2.** Accession numbers, *Thyasira cf. gouldi* OTU 18S rRNA, 28S rRNA and CO1 gene fragments.

OTU	18S	28S	CO1
1	KJ424346	KJ424347	KJ424337, KJ424338, KJ424339
2	KJ424348	KJ424349	KJ424340, KJ424341, KJ424342
3	KJ424350	KJ424351	KJ424343, KJ424344, KJ424345

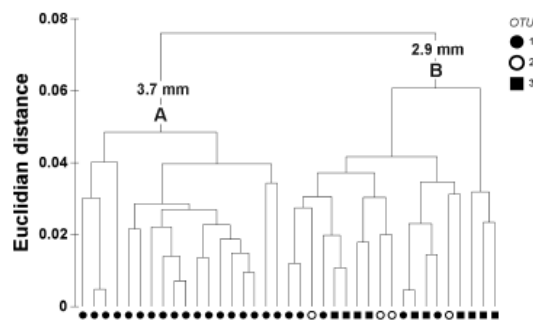
doi:10.1371/journal.pone.0092856.t002

**Table 3.** Distance matrix based on CO1 (667 bp) OTU sequences.

	Between OTUs		Within OTUs
	OTU 1	OTU 2	
OTU 1			0.00071 ± 0.00020 <sup>c</sup>
OTU 2	0.11714 ± 0.01128 <sup>a</sup>		0.00150 ± 0.00082 <sup>c</sup>
OTU 3	0.11779 ± 0.01184 <sup>a</sup>	0.11519 ± 0.01175 <sup>b</sup>	0.01116 ± 0.00227 <sup>d</sup>

d values (averages) were calculated using the 'p-distance' model in MEGA5, treating gaps/missing data with pairwise deletion. Standard error estimates on each comparison are based on 2000 bootstrap replicates. Significant differences (p < 0.01) based on Tukey's HSD post-hoc test are indicated by different letters (<sup>a-d</sup>).

doi:10.1371/journal.pone.0092856.t003



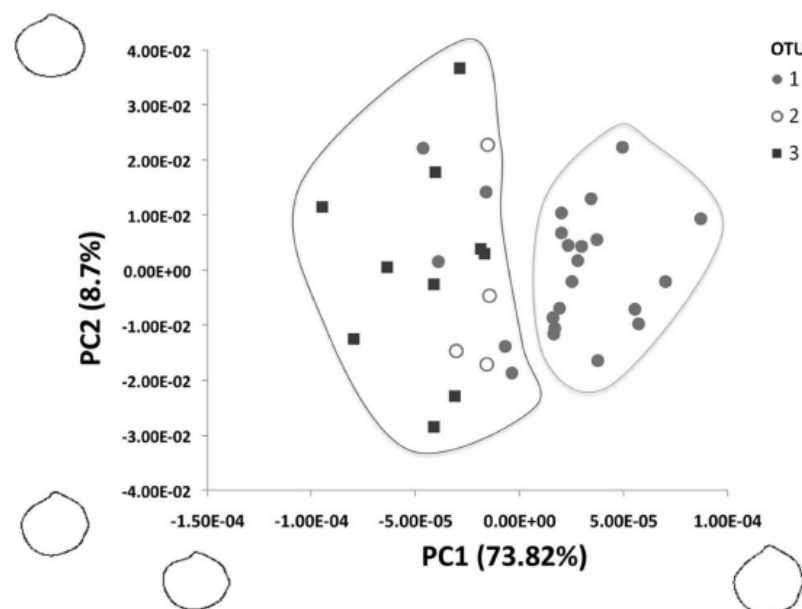
**Figure 3. Cluster analysis of shell outlines (Elliptical Fourier Analysis).** The analysis is based on the first 10 harmonics. Shells form two groups, A and B, which differ significantly in length (indicated above the groups) based on a t-test ( $p < 0.01$ ). Symbols refer to operational taxonomic groups (OTUs) based on CO1, 18S and 28S rRNA sequences. doi:10.1371/journal.pone.0092856.g003

[36]. Comparing reported sizes of symbiotic and asymbiotic thyasirids reveals a similar trend [4].

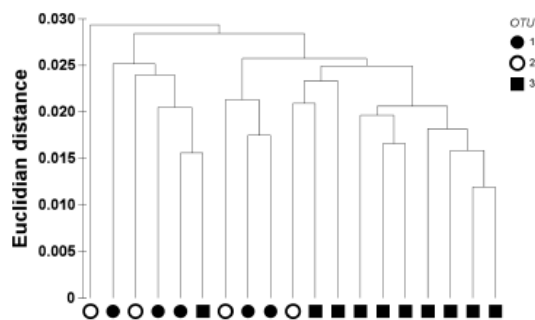
Asymbiotic and symbiotic *Thyasira cf. gouldi* were found in close proximity (i.e., sometimes within the same grab sample); this distribution might be related to sulphide patchiness at the sampling sites. At a cold seep, the distribution of two chemosymbiotic vesicomyids (*Calyptogena kilmeri* & *C. pacifica*) with different sulphide physiology characteristics was related to the fine-scale patchiness of sulphide [39]. Environmental characteristics may also explain the apparent absence of asymbiotic *T. cf. gouldi* at Neddy's Harbour, the shallow site that has coarser sediments, experiences higher temperature and salinity variations (unpublished data) and is subject to dredging (R. Hooper, pers. comm.).

#### Differences in shell characters

Shell shape differed, although imperfectly, between symbiotic and asymbiotic *Thyasira cf. gouldi* specimens. In a first cluster analysis, symbiotic individuals with dorso-ventrally elongated shells (mainly larger specimens) were clearly distinct from other individuals (a mixture of smaller symbiotic and asymbiotic specimens). Smaller symbiotic individuals were similar in dorso-ventral length to asymbiotic specimens, but slight differences in shell outline appeared between those groups. The extent of dorso-ventral elongation of thyasirid shells is thought to be associated with burrowing depth and motility in sediments: species with shells higher than long are deep burrowers while species with shells longer than high remain close to the sediment surface [40]. It



**Figure 4. Shape variation among shells along the first two principal components.** Reconstructed contours show shapes at the extremities of each axis. Symbols refer to operational taxonomic groups (OTUs) based on CO1, 18S and 28S rRNA sequences. doi:10.1371/journal.pone.0092856.g004



**Figure 5. Cluster analysis of a subset of shell outlines (Elliptical Fourier Analysis).** Shells analysed are those from group B in Figure 3. The analysis is based on the first 20 harmonics, excluding the first one. Symbols refer to operational taxonomic groups (OTUs) based on CO1, 18S and 28S rRNA sequences.  
doi:10.1371/journal.pone.0092856.g005

could be advantageous for symbiotic *T. cf. gouldi* to burrow more deeply since the reduced sulphur compounds presumably required for the chemoautotrophic metabolism of their symbionts would be more accessible in deeper sediment layers. Also, the slightly more dorsal location of the ferruginous patch on the shell of symbiotic *T. cf. gouldi* may be associated with a physiological and behavioural adaptation to symbiosis: the location of this patch appears to be associated with the location of inhalant and exhalant currents [40]. Comparing the ventilation, burrowing and sulphur mining [41,42] activities of symbiotic and asymbiotic *T. cf. gouldi* could help explain observed differences in shell characters within this group.

We found no difference in the prodissococonch size of asymbiotic and symbiotic *Thyasira cf. gouldi*. The size range obtained was fairly large, and some uncertainty in sizing is likely due to the angle of the shell when imaging took place. The prodissococonch measures between 205–270  $\mu\text{m}$  in *T. gouldi* individuals ( $N=55$ ) from Norway, Faeroe Islands, New England and Greenland [11], but is smaller in Bonne Bay specimens (180 – 210  $\mu\text{m}$ ,  $N=25$ ). Prodissococonch size can vary with latitude, with size increasing

from south to north [11]. The factors influencing prodissococonch size in thyasirids, and the usefulness of this character in species identification, require further attention.

#### Differences in nuclear and mitochondrial gene sequences

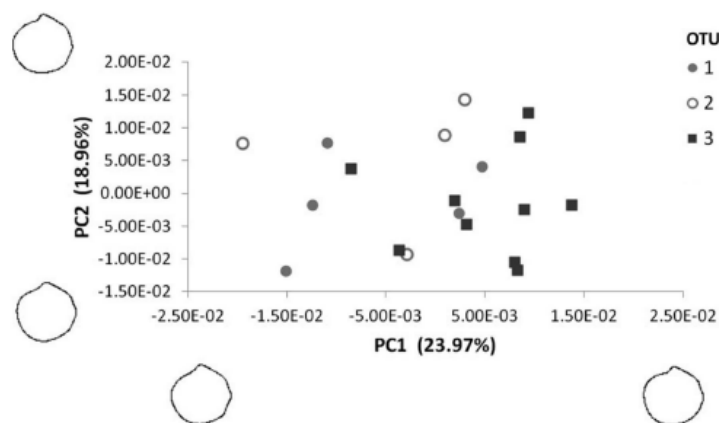
Genes bring further support for the presence of distinct groups among the specimens studied. We identified three OTUs based on nuclear and mitochondrial gene sequences, and evolutionary  $d$  in the CO1 gene sequences between pairs of OTUs was significantly higher than the  $d$  within OTUs suggesting the co-occurrence of three distinct (cryptic) species in Bonne Bay: two symbiotic and one asymbiotic. We are currently unable to distinguish OTUs 1 and 2 based on morphology or ultrastructure. Until these groups are formally described, we retain their OTU designation.

A previous study of four undescribed *Thyasira* sp. individuals reported very low levels of divergence ( $<0.1\%$ ) within the 28S gene fragment and no difference in 18S fragments [43]. In comparison, 18S and 28S genes in *T. gouldi* show a high degree of variation: for example, 18S sequences of *T. gouldi* from the north and south coasts of the UK (AJ581871 and JF899224) have 8 bp discrepancies within 956 bp, and 28S sequences from those same sites differ by 12 bp out of 573. Therefore, the *T. gouldi* lineage appears highly divergent.

#### Possible species complex within *Thyasira gouldi*

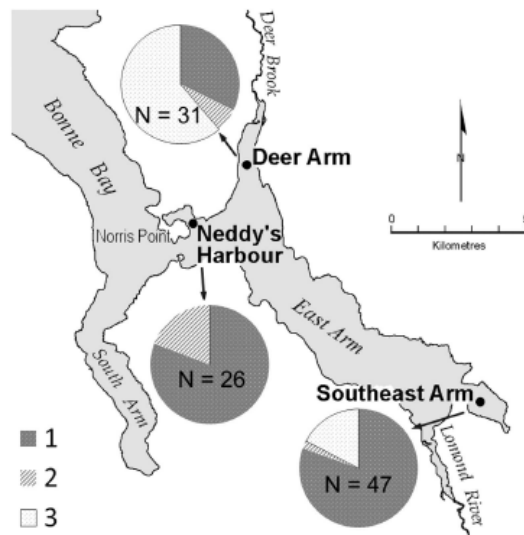
Because of: 1) consistent differences in shell outline, gill characters and gene sequences between symbiotic and asymbiotic Bonne Bay individuals; 2) the presumed ampho-Atlantic geographic range of this larval brooding [44] species; 3) the considerable variation in shell outline reported for this species [11]; 4) the high degree of variability in nuclear and mitochondrial gene sequences between specimens from different locations, we argue that *Thyasira gouldi* forms a cryptic species complex. As most of the information currently available on type specimens consists of shell characters (descriptions of internal anatomy and gene sequences only apply to specimens from the eastern Atlantic), a detailed examination and genetic analysis of material from the type location will be required to properly delineate species within this group.

Cryptic, sibling, or incipient species complexes [45–48]) have been discovered in the chemosymbiotic bivalve families Vesico-



**Figure 6. Shape variation in a subset of shells along the first two principal components.** Shells analysed belong to group B in Figure 3. Reconstructed contours show shapes at the extremities of each axis. Symbols refer to operational taxonomic groups (OTUs) based on CO1, 18S and 28S rRNA sequences.  
doi:10.1371/journal.pone.0092856.g006





**Figure 7. Distribution of *Thyasira cf. gouldi* groups in Bonne Bay, NL.** Charts at each location show relative proportions of each OTU (1–3) and total number of individuals (N) used in the molecular analysis. Sampling sites are Deer Arm, Neddy's Harbour, and Southeast Arm. doi:10.1371/journal.pone.0092856.g007

myidae [49,50] and Lucinidae [51], but not in the Thyasiridae. However, unlike in these cases, the *T. gouldi* complex is remarkable as it comprises both symbiotic and asymbiotic species. A clear relationship between OTU and symbiont presence was found, as all OTU 1 and OTU 2 individuals were symbiotic and had elongated (type 3) gill filaments, while all OTU 3 individuals lacked symbionts and had short (type 2) gill filaments. Thus, symbiosis may constitute a particularly strong evolutionary driving force in this group, with symbiont presence and gill structure being subject to change over relatively short evolutionary timescales. Further phylogenetic analyses including *T. gouldi* specimens from other sites should help elucidate the sequence of symbiont appearance (or loss) within this group. The relatively rapid switch between symbiotic and asymbiotic states observed here suggests that symbionts are not vertically transmitted but are acquired from

the sedimentary environment (i.e. horizontally or environmentally) at a juvenile stage, as described in other invertebrates [52,53]. Under this scenario, and considering that gill filament elongation is triggered by symbionts, it is conceivable that groups of thyasirids subjected to different environmental pressures would either associate with symbionts or not. Reproductive isolation in different groups would be facilitated by the small-scale dispersion of brooded juveniles and eventually lead to speciation.

## Conclusions

Many gaps remain in understanding the evolutionary history and distribution patterns within the Thyasiridae, especially since published gene sequences for most thyasirid species represent a single geographic location, and possibly a single individual. Species-level identification of thyasirids based on shell features is challenging because of the paucity of clear diagnostic characters that are often confounded by convergent or parallel evolution [25], and the presumed high intraspecific variation in shell form [1]. The wide distribution of many thyasirids should be re-assessed in light of the differences observed here in gene sequences from apparent conspecifics spanning the Atlantic Ocean. The striking differences we show in gill filament morphology underscore the relevance of this organ as a species diagnostic character [40]; symbiosis can directly affect gill morphology and have significant (and possibly rapid) ecological and evolutionary consequences for the host.

## Acknowledgments

R Hooper assisted with thyasirid collection at the Bonne Bay Marine Station. E Perry and E Yaskowiak helped with work performed at the CREAT Genomics and Proteomics Facility (MUN), and C Weland performed COI gene sequencing at the Canadian Centre for DNA Barcoding (University of Guelph). S Tucker and K Williams (EM Unit, Faculty of Medicine, MUN) assisted with Transmission Electron Microscopy. L Men and M Shaffer (CREAT Bio-imaging cluster and Micro-analysis Facility, MUN) operated the scanning electron microscope. J. Hershey provided useful discussions on shape analysis. We thank two anonymous reviewers for constructive comments.

## Author Contributions

Conceived and designed the experiments: SCD RTB. Performed the experiments: JRL FS RTB. Analyzed the data: SCD RTB JRL FS. Contributed reagents/materials/analysis tools: SCD. Wrote the paper: SCD RTB JRL FS.

## References

- Taylor JD, Williams ST, Glover EA (2007) Evolutionary relationships of the bivalve family Thyasiridae (Mollusca: Bivalvia), monophyly and superfamily status. *J Mar Biol Assoc UK* 87: 565–574.
- Duperron S, Gaudron SM, Rodrigues CF, Cunha MR, Decker C, et al. (2012) An overview of chemosynthetic symbioses in bivalves from the North Atlantic and Mediterranean Sea. *Biogeosciences Discuss* 9: 16815–16875.
- Southward EC (1986) Gill symbionts in Thyasiridae and other bivalve molluscs. *J Mar Biol Assoc U K* 66: 889–914.
- Dufour SC (2005) Gill anatomy and the evolution of symbiosis in the bivalve family Thyasiridae. *Biol Bull* 208: 200–212.
- Dando PR, Spiro B (1993) Varying nutritional dependence of the thyasirid bivalves *Thyasira sarsi* and *T. equalis* on chemoautotrophic symbiotic bacteria, demonstrated by isotope ratios of tissue carbon and shell carbonate. *Mar Ecol Prog Ser* 92: 151–158.
- Fujiwara K, Kato C, Masui N, Fujikura K, Kojima S (2001) Dual symbiosis in the cold-seep thyasirid clam *Maorithyas hadaki* from the hadal zone in the Japan Trench, Western Pacific. *Mar Ecol Prog Ser* 214: 151–159.
- Dando PR, Southward AJ (1986) Chemoautotrophy in bivalve molluscs of the genus *Thyasira*. *J Mar Biol Assoc U K* 66: 915–929.
- Le Penec M, Diouris M, Herry A (1988) Endocytosis and lysis of bacteria in gill epithelium of *Bathymodiolus thermophilus*, *Thyasira flexuosa* and *Lucinella dioriana* (Bivalve, Molluscs). *J Shellfish Res* 7: 483–489.
- Dufour SC, Felbeck H (2006) Symbiont abundance in thyasirids (Bivalvia) is related to particulate food and sulphide availability. *Mar Ecol Prog Ser* 320: 185–194.
- Coan EV, Scott PV, Bernard FR (2000) Bivalve seashells of western North America: marine bivalve mollusks from arctic Alaska to Baja California. Santa Barbara: Santa Barbara Museum of Natural History Monographs. 764 p.
- Oliver PG, Killen IJ (2002) The Thyasiridae (Mollusca: Bivalvia) of the British continental shelf and North Sea oil fields: an identification manual. *Wales: Studies in Marine Biodiversity and Systematics from the National Museum of Wales*. 73 p.
- Ockemann KW (1958) The zoology of East Greenland: Marine Lamellibranchiata. *Medd Grøn* 122: 1–256.
- Włodarska-Kowalczyk M (2007) Molluscs in Kongsfjorden (Spitsbergen, Svalbard): A species list and patterns of distribution and diversity. *Polar Res* 26: 48–63.
- Gould AA (1841) Report on the Invertebrata of Massachusetts, comprising the Mollusca, Crustacea, Annelida and Radiata. Cambridge: Folsom, Wells and Thurston. 373 p.
- Gould AA, Binney WG (1870) Report on the invertebrata of Massachusetts comprising the Mollusca, 2nd edition. Boston: Wright and Potter. 524 p.



16. Distel DL, Wood AP (1992) Characterization of the gill symbiont of *Thyasira fleasosa* (Thyasiridae: Bivalvia) by use of polymerase chain reaction and 16S rRNA sequence analysis. *J Bacteriol* 174: 6317–6320.
17. Conan GY, Starr M, Comeau M, Theriault JC, Maynou FX, et al. (1996) Life history strategies, recruitment fluctuations, and management of the Bonne Bay Fjord Atlantic snow crab (*Chionoecetes opilio*). In: Baxter R, editor. Proceedings of the international symposium on the biology, management, and economics of crabs from high latitude habitats. Lowell: Alaska Sea Grant College Program Report. pp. 59–97.
18. Crampton JS (1995) Elliptic Fourier shape analysis of fossil bivalves: some practical considerations. *Lethaia* 28: 179–186.
19. Delgado-Gonzalo R, Thévenaz P, Sedamantula CS, Unser M (2012) Snakes with an ellipse-reproducing property. *IEEE T Image Process* 21: 1258–1271.
20. Abramoff MD, Magalhaes PJ, Ram SJ (2004) Image processing with ImageJ. *Biophotonics Int* 11: 36–42.
21. Iwata H, Ukai Y (2002) SHAPE: A computer program package for quantitative evaluation of biological shapes based on Elliptic Fourier descriptors. *J Heredity* 93: 384–385.
22. Clarke KR, Gorley RN (2006) PRIMER v6: User manual/tutorial. Plymouth: PRIMER-E. 192 p.
23. Distel DL, Amin M, Burgoyne A, Linton E, Mamangkey G, et al. (2011) Molecular phylogeny of Pholadoidea Lamarck, 1809 supports a single origin for xylophagy (wood feeding) and xylophagous bacterial endosymbiosis in Bivalvia. *Mol Phylogenet Evol* 61: 245–254.
24. Williams ST, Taylor JD, Glover EA (2004) Molecular phylogeny of the Lucinoidea (Bivalvia): non-monophyly and separate acquisition of bacterial chemosymbiosis. *J Mollus Stud* 70: 187–202.
25. Steiner G, Hammer S (2000). Molecular phylogeny of the Bivalvia inferred from 18S rDNA sequences with particular reference to the Pteriomorpha. In: Harper EM, Taylor JD, Crame JA, editors. The evolutionary biology of the Bivalvia. London: Geological Society Special Publications. pp. 11–29.
26. Winnepeinnicks B, Reid DG, Backeljau T (1998) Performance of 18S rRNA in littorinid phylogeny (Gastropoda: Caenogastropoda). *J Mol Evol* 47: 586–596.
27. Williams ST, Reid DG, Littlewood DTJ (2003) A molecular phylogeny of the Littorininae (Gastropoda: Littorinidae): unequal evolutionary rates, morphological parallelism, and biogeography of the southern ocean. *Mol Phylogenet Evol* 28: 60–86.
28. Littlewood DTJ, Curini-Galletti M, Herniou EA (2000) The interrelationships of Proseriata (Platyhelminthes: Seriata) tested with molecules and morphology. *Mol Phylogenet Evol* 16: 449–466.
29. Layton K (2012) Examining patterns of genetic variation in Canadian marine molluscs through DNA barcodes. MSc Thesis, University of Guelph, Canada.
30. Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990). Basic local alignment search tool. *J Mol Biol* 215: 403–410.
31. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, et al. (2011) MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol* 28: 2731–2739.
32. Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res* 22: 4673–4680.
33. Castresana J (2000) Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. *Mol Biol Evol* 17: 540–552.
34. Neumann D, Kappes H (2003) On the growth of bivalve gills initiated from a lobule-producing budding zone. *Biol Bull* 205: 73–82.
35. Cammuel R, Beninger PG, McCombie G, Boudry P (2009) Gill development and its functional and evolutionary implications in the blue mussel *Mytilus edulis* (Bivalvia: Mytilidae). *Biol Bull* 217: 173–188.
36. Gros O, Frenkiel I, Moueza M (1998) Gill filament differentiation and experimental colonization by symbiotic bacteria in aposymbiotic juveniles of *Codakia orbicularis* (Bivalvia: Lucinidae). *Inv Rep Dev* 34: 219–231.
37. Montgomery MK, McFall-Ngai M (1994) Bacterial symbionts induce host organ morphogenesis during early postembryonic development of the squid *Euprymna scolopes*. *Development* 120: 1719–1729.
38. Cheesman SE, Neal JT, Mitte E, Serebicki BM, Guillemin K (2011) Epithelial cell proliferation in the developing zebrafish intestine is regulated by the Wnt pathway and microbial signaling via Myd88. *Proc Nat Acad Sci U S A* 108 Suppl 1: 4570–4577.
39. Goffredi SK, Bany JP (2002) Species-specific variation in sulfide physiology between closely related Vesicomid clams. *Mar Ecol Prog Ser* 225: 227–238.
40. Payne CM, Allen JA (1991) The morphology of deep-sea Thyasiridae (Mollusca: Bivalvia) from the Atlantic Ocean. *Phil Trans Biol Sci* 334: 481–562.
41. Dufour SC, Felbeck H (2003) Sulphide mining by the superextensive foot of symbiotic thyasirid bivalves. *Nature* 426: 65–67.
42. Dando PR, Southward AJ, Southward EC (2004) Rates of sediment sulphide oxidation by the bivalve mollusc *Thyasira sarsi*. *Mar Ecol Prog Ser* 280: 181–187.
43. Brissac T, Rodrigues CF, Gros O, Duperron S (2011). Characterization of bacterial symbioses in *Mytilus* sp. (Bivalvia: Lucinidae) and *Thyasira* sp. (Bivalvia: Thyasiridae) from a cold seep in the Eastern Mediterranean: identification of two bivalves and their bacterial symbionts. *Mar Ecol* 32: 198–210.
44. Blacknell WM, Ansell AD (1974) The direct development of bivalve *Thyasira gouldi* (Philippi). *Thalassia Jugoslav* 10: 23–43.
45. Knowlton N (1993) Sibling species in the sea. *Annu Rev Ecol Syst* 24: 189–216.
46. Gardner JPA (1994) The *Mytilus edulis* species complex in southwest England: multi-locus heterozygosity, background genotype and a fitness correlate. *Biochem Syst Ecol* 22: 1–11.
47. Gardner JPA (1996) The *Mytilus edulis* species complex in southwest England: effects of hybridization and introgression upon interlocus associations and morphometric variation. *Mar Biol* 125: 385–399.
48. Mikkelsen NT (2011) Speciation in modern marine bivalves (Mollusca: Bivalvia): insights from the published record. *Am Malacol Bull* 29: 217–245.
49. Vrijenhoek RC, Schutz SJ, Gustafson RG, Lutz RA (1994) Cryptic species of deep-sea clams (Mollusca: Bivalvia: Vesicomidae) from hydrothermal vent and cold-water seep environments. *Deep-Sea Res* 41: 1171–1189.
50. Goffredi SK, Hurtado LA, Hallam SJ, Vrijenhoek RC (2003) Evolutionary relationships of deep-sea vent and cold seep clams (Mollusca: Vesicomidae) of the “Pacifica/Lepta” species complex. *Mar Biol* 142: 311–320.
51. Taylor JD, Glover EA (2005) Cryptic diversity of chemosymbiotic bivalves: a systematic revision of worldwide *Anadenia* (Mollusca: Bivalvia: Lucinidae). *Syst Biodivers* 3: 281–338.
52. Gros O, Darrasse A, Durand P, Frenkiel I, Moueza M (1996) Environmental transmission of a sulfur-oxidizing bacterial gill endosymbiont in the tropical lucinid bivalve *Codakia orbicularis*. *Appl Environ Microb* 62: 2324–2330.
53. Nusbaumer AD, Fisher CR, Bright M (2006) Horizontal endosymbiont transmission in hydrothermal vent tubeworms. *Nature* 441: 35–348.

**Appendix 2:** Dufour, S. C., Laurich, J. R., Batstone, R. T., McCuaig, B., Elliot, A., & Poduska, K. M. (2014). Magnetosome-containing bacteria living as symbionts of bivalves. *The ISME Journal*.doi: 10.1038/ismej.2014.93.

## ORIGINAL ARTICLE

# Magnetosome-containing bacteria living as symbionts of bivalves

Suzanne C Dufour<sup>1</sup>, Jason R Laurich<sup>1</sup>, Rebecca T Batstone<sup>1,3</sup>, Bonita McCuaig<sup>1</sup>, Alexander Elliott<sup>1,4</sup> and Kristin M Poduska<sup>2</sup>

<sup>1</sup>Department of Biology, Memorial University of Newfoundland, St John's, Newfoundland A1B 3X9, Canada and <sup>2</sup>Department of Physics and Physical Oceanography, Memorial University of Newfoundland, St John's, Newfoundland A1B 3X7, Canada

Bacteria containing magnetosomes (protein-bound nanoparticles of magnetite or greigite) are common to many sedimentary habitats, but have never been found before to live within another organism. Here, we show that octahedral inclusions in the extracellular symbionts of the marine bivalve *Thyasira* cf. *gouldi* contain iron, can exhibit magnetic contrast and are most likely magnetosomes. Based on 16S rRNA sequence analysis, *T. cf. gouldi* symbionts group with symbiotic and free-living sulfur-oxidizing, chemolithoautotrophic gammaproteobacteria, including the symbionts of other thyasirids. *T. cf. gouldi* symbionts occur both among the microvilli of gill epithelial cells and in sediments surrounding the bivalves, and are therefore facultative. We propose that free-living *T. cf. gouldi* symbionts use magnetotaxis as a means of locating the oxic–anoxic interface, an optimal microhabitat for chemolithoautotrophy. *T. cf. gouldi* could acquire their symbionts from near-burrow sediments (where oxic–anoxic interfaces likely develop due to the host's bioirrigating behavior) using their superextensible feet, which could transfer symbionts to gill surfaces upon retraction into the mantle cavity. Once associated with their host, however, symbionts need not maintain structures for magnetotaxis as the host makes oxygen and reduced sulfur available via bioirrigation and sulfur-mining behaviors. Indeed, we show that within the host, symbionts lose the integrity of their magnetosome chain (and possibly their flagellum). Symbionts are eventually endocytosed and digested in host epithelial cells, and magnetosomes accumulate in host cytoplasm. Both host and symbiont behaviors appear important to symbiosis establishment in thyasirids.

The ISME Journal advance online publication, 10 June 2014; doi:10.1038/ismej.2014.93

## Introduction

A taxonomically and ecologically diverse group of free-living bacteria are magnetotactic: their cytoplasm contains one or more chains of protein-bound, biomineralized magnetite or greigite ('magnetosomes'), producing a magnetic dipole within the cell (Lefèvre and Bazylinski, 2013). Magnetotactic bacteria can align with the Earth's magnetic field and move along geomagnetic field lines using their flagella. Many magnetotactic bacteria are chemolithoautotrophic microaerophiles that use magnetotaxis to locate the oxic–anoxic interface (OAI) within sediments, a favorable environment for them

due to the proximity of reduced compounds (for example, hydrogen sulfide) and oxygen (Lefèvre and Bazylinski, 2013).

Although most chemolithoautotrophic bacteria have a free-living existence, some form obligate or facultative symbioses with marine invertebrates (Dubilier *et al.*, 2008). The sulfur-oxidizing symbionts of thyasirid bivalves are among the few examples of chemosymbionts that are likely facultative (that is, capable of living both in hosts and in the outside environment), given that they are located outside rather than inside host gill cells (Southward, 1986; Dufour, 2005) and that, in at least one species (*Thyasira* n. sp. Guinness), host individuals can associate with different symbiont phenotypes, suggesting acquisition from surrounding sediments (Duperron *et al.*, 2012a). Nevertheless, bacteria that associate with thyasirids are considered to be symbionts and not merely microbes trapped on gill epithelia as water circulates within the pallial cavity, because individual hosts associate with a single dominant phenotype of sulfur-oxidizing bacteria (Duperron *et al.*, 2012b). Intriguingly, transmission electron micrographs of the gills of at

Correspondence: SC Dufour, Department of Biology, Memorial University of Newfoundland, 232 Elizabeth St, St John's, Newfoundland A1B 3X9, Canada.  
E-mail: sdufour@mun.ca

<sup>3</sup>Current address: Department of Ecology and Evolutionary Biology, University of Toronto, Toronto, Ontario, Canada M5S 3G5.

<sup>4</sup>Current address: Department of Biological Sciences, University of Calgary, Calgary, Alberta, Canada T2N 1N4.

Received 20 February 2014; revised 6 May 2014; accepted 8 May 2014



least four thyasirid species reveal that their symbionts contain abundant electron-dense inclusions, interpreted as being viruses (Southward and Southward, 1991; Brissac *et al.*, 2011; Duperron *et al.*, 2012b).

*Thyasira cf. gouldi* from Bonne Bay were recently described as forming a cryptic complex in which there are two symbiotic operational taxonomic units (OTUs 1 and 2) and an asymbiotic OTU (3) of bivalves, which vary slightly in shell shape (Batstone *et al.*, 2014). Similar to other thyasirids (Southward, 1986; Dufour, 2005), the symbionts of *T. cf. gouldi* OTUs 1 and 2 are maintained at the surface of gill epithelial cells within extracellular 'pockets' bounded by cytoplasmic extensions of host cells that often bear microvilli (Batstone *et al.*, 2014). Symbiotic thyasirids meet part of their nutritional requirements by assimilating carbon fixed by symbionts (Dando and Spiro, 1993), and electron micrographs show that symbionts are periodically engulfed within gill epithelial cells, a process that results in whorls of lysed bacterial products in host cells (Southward, 1986; Dufour, 2005). As thyasirid symbionts are held among gill epithelial cells, they likely benefit from host bioirrigation and sulfur-mining behaviors, which give them access to oxygen and reduced sulfur (Dufour and Felbeck, 2003; Dando *et al.*, 2004).

Here, we use multiple lines of evidence to show that inclusions in the symbionts of *Thyasira cf. gouldi* from Bonne Bay, Canada contain iron (most likely iron sulfide), can show magnetic contrast and, in some cases, are arranged in distinct chains; this is the first report of invertebrates forming associations with bacteria that contain magnetosomes.

## Materials and methods

### Sample collection and dissection

Sediments were collected from three sites (Deer Arm: 30 m depth; Southeast Arm: 30 m depth; and Neddy's Harbour: 15 m depth) in the fjord of Bonne Bay, Newfoundland, Canada, during multiple collection trips between September 2009 and November 2013. Sediments were sieved on a 1 mm mesh and symbiotic individuals of *Thyasira cf. gouldi* were retained; symbiotic and asymbiotic *T. cf. gouldi* were sorted on the basis of shell shape and gill morphology upon dissection (Batstone *et al.*, 2014). We could not distinguish between the two symbiotic OTUs (which differ in 18S, 28S and CO1 gene sequences but not in morphological characters), so all specimens analyzed herein consist of either OTU 1 (the most common type) or OTU 2.

Upon dissection, the color of the gills (which varied from pale-pink to black) was noted. Gills of dissected individuals underwent different treatments: some were rinsed in distilled water and individually placed in vials of 95% ethanol for DNA extraction and analysis (see below), while others

were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (24 h) for either transmission electron microscopy (TEM), selected area electron diffraction (SAED), histological staining or environmental scanning electron microscopy (ESEM) and elemental analysis. Symbionts from the gill of one individual were isolated immediately for atomic force microscopy/magnetic force microscopy (AFM/MFM), as described below.

### TEM

To observe symbionts, gills were post-fixed in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer (1 h), dehydrated in an ascending ethanol series and embedded in TAAB 812 resin (Canemco, Canton de Gore, QC, Canada). Ultra-thin (60 nm) transverse gill sections were post-stained with uranyl acetate and lead citrate, and imaged using a Philips 300 transmission electron microscope (Tokyo, Japan).

### Histological staining for iron

A prussian blue histological staining protocol (Sheehan and Hrapchak, 1980) was used to localize iron in the gills of 11 *Thyasira cf. gouldi* specimens. Gills were dehydrated in an ascending ethanol series (without post-fixation in osmium), embedded in paraffin and sectioned (5  $\mu$ m thick). Deparaffinized and hydrated transverse sections of gill filaments were immersed for 20 min in a freshly prepared solution of 10% aqueous hydrochloric acid and 5% aqueous potassium ferrocyanide, rinsed three times in distilled water and counterstained with Hematoxylin (1 min) before dehydration, cover slipping and imaging with a Zeiss light microscope (Munich, Germany).

### Elemental analysis

To investigate whether symbiont inclusions contained iron, we mounted thin (1  $\mu$ m) resin sections of one gill (post-fixed in 1% osmium tetroxide) on stubs for ESEM and elemental analysis. We used a standard (solid state) backscattered electron detector in an FEI Quanta 650F ESEM (Eindhoven, The Netherlands) to observe and image symbiont inclusions in combination with a Bruker XFlash SSD 5030 X-ray detector (Berlin, Germany) for elemental analysis.

### AFM/MFM

We used AFM/MFM to determine whether inclusions are magnetic, as in (Proksch *et al.*, 1995). Symbionts were isolated from a single gill using a Percoll cushion (Distel and Felbeck, 1988), smeared on a glass slide, air-dried and magnetized by placing a magnet perpendicular to the plane of the slide for tens of seconds. We used an Asylum Research MFP-3D Atomic Force Microscope (Santa Barbara, CA, USA), operating in an interleaved AC/lift mode, for

all imaging. A Co-Cr coated tip (Mikromasch NSC36, Wetzlar, Germany) was magnetized perpendicular to the cantilever plane before imaging, and samples were magnetized in the plane of the slide. Lift heights of 35–150 nm were tested for magnetic contrast imaging.

#### SAED

The crystal structure of inclusions was studied using SAED. Resin-embedded gill sections were coated with carbon and then examined in a FEI Titan low base TEM (Eindhoven, The Netherlands) operated at 300 kV. Electron diffraction patterns were obtained by focusing the beam on either one or two inclusions at a time, and d-spacings were determined from these patterns.

#### DNA extraction and amplification

To identify symbionts, DNA was extracted from the gills of three *Thyasira* cf. *gouldi* specimens (following confirmation of symbiont inclusions in the other gill using TEM) using the QIAgen DNeasy Blood and Tissue kit (Hilden, Germany), following the spin-column protocol for animal tissues. We performed PCRs using a universal primer set (27F and 1492R; Lane, 1991), the Promega Master Mix (Promega, Madison, WI, USA) and the following thermocycler conditions: initial denaturation at 94 °C for 4 min, 35 cycles of denaturation at 94 °C for 30 s, annealing (30 s) at 50 °C and elongation at 72 °C for 2 min, with a final elongation at 72 °C for 5 min. We then filtered all amplified products using Acro-Pro 100K-Omega filters (Pall Life Sciences, Port Washington, NY, USA), performed sequencing reactions using BigDye Terminator v3.1 Cycle Sequencing Ready Reaction Mix (Applied Biosystems, Foster City, CA, USA) and electrophoresed on an Applied Biosystems 3730 DNA Analyzer automated capillary sequencer running Sequencing Analysis v. 5.2 Software (Applied Biosystems).

We also sought to determine which form of the Calvin–Benson–Bassham enzyme RuBisCO was present in *Thyasira* cf. *gouldi* symbionts, as several magnetotactic bacteria had previously been shown to contain RuBisCO form II (*cbbM*) rather than form I (*cbbL*) (Bazylnski and Williams, 2006). To do so, we performed PCR with primer sets and conditions specific for RuBisCO forms I and II (Elsaied and Naganuma, 2001).

#### Molecular phylogenetic analysis and reconstruction

We used Sequencher (v. 5.2.3, Gene Codes, Ann Arbor, MI, USA) to edit the forward and reverse 16S sequences and used BLASTn (Altschul et al., 1990) to identify the most closely related sequences in GenBank. We then aligned the consensus sequence with various other published sequences confirmed as magnetotactic bacteria or close relatives using

Clustal W in MEGA5 (Tamura et al., 2011). We constructed a phylogenetic tree using MrBayes (v. 3.2.2; Huelsenbeck and Ronquist, 2001) with GTR as the substitution model, rate variation set to gamma distribution with four discrete categories and allowance for invariant sites. Data sets were run twice, with initial settings of 10 000 000 generations each and a sampling frequency of once every 100 generations; however, once the convergence diagnostics hit the target value of 0.01, the analysis stopped resulting in a total of 442 trees read. The consensus tree was based on the 332 trees that were actually sampled. Support values at each node represent posterior probabilities calculated in MrBayes.

#### Detection of free-living symbionts in sediments

To determine whether free-living symbionts exist in sediments from the *Thyasira* cf. *gouldi* habitat, we first used fluorescence *in situ* hybridization (FISH) to label bacteria extracted from sediment samples, as in (Gros et al., 2003). Based on the 16S rRNA sequence obtained, a 20-base oligonucleotide probe specific for *T. cf. gouldi* symbionts (5'-GCTCACC AAGGCAGCGATCC-3', melting  $T = 57.97$  °C) was designed using the Arb 5.5 probe design tool (Wolfgang et al., 2004) and the gammaproteobacterial 16S rRNA Silva database as of 10 January 2013. Oligonucleotide probes were labeled with fluorescein at the 5' end. Although other sequences in GenBank were identical to the symbiont probe sequence, closest matches belonged to bacteria from terrestrial or hypersaline environments; therefore, the probe was considered to be specific to the *T. cf. gouldi* symbiont, at least within its native sediments (out of several possible probes, we selected the one that produced the least non-specific hits). To reduce the likelihood of unspecific binding, an unlabeled competitive probe with one-base difference (5'-GCT CACCAAGGCAACGATCC-3', melting  $T = 55.19$  °C) was used concurrently with the labeled probe in FISH assays (this approach was shown by Manz et al., 1992 to drastically reduce non-specific binding). To test probe specificity and determine appropriate hybridization temperatures and formamide concentrations, symbionts extracted from a homogenized gill were filtered on a 0.22-µm black polycarbonate filter and hybridization was attempted under different conditions; binding was successful at 46 °C and with 40% formamide. Labeling with a fluorescein-labeled version of the competitive probe did not occur under these conditions.

Sediment cores (1 cm diameter) were collected from grab samples at the study sites, and subdivided into 0–2-, 2–5- and 5–10-cm-depth fractions. Samples were stored at –20 °C in equal volumes of fixative (4% formaldehyde in the following buffer: 40 g l<sup>-1</sup> Borax in sterile 0.22 µm filtered seawater, pH 8.8), in 15-ml centrifuge tubes. Bacteria were



extracted from 1.5 ml of sediments by deflocculating using 250  $\mu$ l of 100 mM sodium pyrophosphate and 3.25 ml of the above-mentioned buffer, followed by three cycles of sonication (10 s), and vortexing (30 s), and a final resting period of 15 min with periodic vortexing. Centrifuge tubes were then placed upright until most sediments appeared to have settled (1–3 min), and 1 ml of supernatant was filtered onto black 0.22  $\mu$ m polycarbonate filters. Bacterial cells were permeabilized with 95% ethanol (1 min), placed on grease-covered slides and incubated with 50 ng probe in 38  $\mu$ l hybridization buffer (40% formamide, 0.9 M NaCl, 20 mM Tris/HCl pH 7.4, 0.01% sodium dodecyl sulfate) at 46 °C for 2 h. Filters were then placed into 1.5 ml pre-warmed wash buffer (70 mM NaCl, 20 mM Tris/HCl pH 7.4, 5 mM EDTA, 0.01% sodium dodecyl sulfate) for 10 min, then rinsed with distilled water, dried and mounted on a slide using Permafluor for observation using a Zeiss Axio Imager A2 (Munich, Germany) with appropriate filter set. A sample of bacteria filtered from overlying seawater from the sampling site was used as a negative control; no fluorescent labeling occurred on these filters. Other filters containing sediment-extracted bacteria that had not gone through the hybridization process were tested for autofluorescence using the same filter set used for fluorescein detection; only very faint background fluorescence was noted on these filters.

Further confirmation of the presence of free-living forms of *Thyasira cf. gouldi* symbionts was obtained by searching for matching 16S sequence fragments among environmental DNA amplicons. Sediment samples from Deer Arm and Southeast Arm were collected in August 2013. Subsamples underwent total DNA extraction using the Xpedition Soil/Fecal DNA Miniprep kit (Zymo Research, Irvine, CA, USA) following the manufacturer's protocol for soil samples. PCR was then performed using barcoded (IonXpress, Life Technologies, South San Francisco, CA, USA) universal microbial primers targeting the hypervariable V6 region of the 16S rRNA gene developed by the Lang Lab of Memorial University, St. John's, NL, Canada (modified from Huber *et al.*, 2007). Reverse primers (5'-CGACRRCCATGCANCA CCT-3') contained the barcode while forward primers (an equal mix of: 5'-CTAACCGANGAACC-TYACC-3', 5'-ATACGCGARGAACCTTACC-3', 5'-CNACGCGAAGAACCTTANC-3' and 5'-CAACGCGMARACCTTACC-3') contained the Ion Torrent adapter sequences (for simplicity, the barcode and adapter sequences are not shown). PCRs were run using Phusion High Fidelity Polymerase and High Fidelity Buffer (New England Biolabs, Ipswich, MA, USA), and dNTPs used in standard concentrations. Thermocycler settings were as follows: 98 °C for 30 s, 30 cycles of 98 °C for 10 s, 72 °C for 30 s, followed by 72 °C for 2 min.

The PCR product was cleaned and prepared for sequencing on the Ion Torrent PGM (Life Technologies) following the protocol provided

in the Ion PGM Template OT2 200 Kit and Ion PGM Sequencing 200 Kit v2. An Ion 314 v2 chip was used for sequencing. Runs were cleaned and filtered using the FastQC suite. Raw reads were trimmed at both ends to obtain a quality score >20. These were then aligned to the known 16S sequence of the *Thyasira cf. gouldi* symbiont as well as close relatives using the Torrent Mapping Alignment Program map1 command. Changes to the default settings included only the best alignments being accepted (-a 0) and an increase from the default 10% misalignment allowance to a 2% misalignment allowance (map1 -max-misalignment 0). The mapping results were then visualized using Integrative Genomics Viewer (Robinson *et al.*, 2011) and manually checked for misalignments.

## Results

### TEM and histology

Transmission electron micrographs of all symbiotic *Thyasira cf. gouldi* gills examined ( $N > 80$  individuals from various dates and from the three sampling sites) revealed numerous electron-dense octahedral inclusions within the cytoplasm of symbionts; in some cases, these inclusions formed the characteristic chains of magnetotactic bacteria (Figure 1a). In most sectioned bacteria, inclusions did not form a chain but were more or less aggregated in the bacterial cytoplasm (Figure 1b). Inclusions in chains measured  $72.9 \pm 28.8$  nm ( $N = 20$  particles) and were more electron dense than inclusions not forming chains ( $55.7 \pm 6.0$  nm;  $N = 20$  particles).

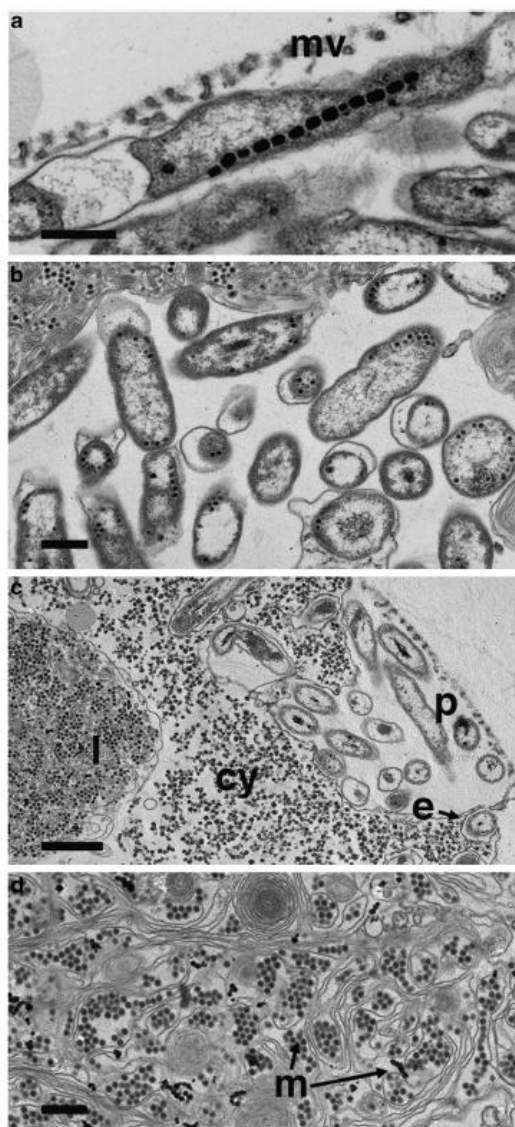
Evidence of the endocytosis and digestion of symbionts by host cells (with the formation of characteristic whorls; Figure 1c) was visible in many gills. Symbiont inclusions resist host digestion and accumulate in whorls (size:  $56.0 \pm 6.3$  nm;  $N = 20$  particles) and in host cytoplasm (size:  $77.4 \pm 11.1$  nm;  $N = 20$  particles; Figure 1d). The abundance of bacterial inclusions varied widely among host specimens. Gill color was related to the overall abundance of inclusions, particularly in host cytoplasm: pink gills contained fewer inclusions than darker gills (based on general observations made on all the gills examined).

The cytoplasm of the bacteriocytes of five specimens (out of 11) stained intensely with prussian blue, revealing the presence of iron (Figure 2a). All specimens that were prussian blue positive had either dark or black gills.

### Elemental analysis of inclusions

With ESEM, cellular structure could be observed in backscatter mode, and bacterial inclusions, sometimes forming chains, were visible (Figure 2b). X-ray spectra revealed the presence of iron in those structures (Figure 2c), but not in adjacent tissue.





**Figure 1** Transmission electron micrographs of symbionts associated with gill epithelial cells of *Thyasira cf. gouldi*. (a) Symbiont with a chain of magnetosomes, located close to the microvillar boundary (mv) of the bacteriocyte. Scale bar, 500 nm. (b) Multiple symbionts, with magnetosomes not organized in a chain. Scale bar, 500 nm. (c) View of symbionts in an extracellular 'pocket' (p), with some undergoing endocytosis (e). A large aggregate of lysed products of symbiont digestion (l), including magnetosomes, is visible in the host cytoplasm. Dark inclusions in the host cytoplasm (cy) may be the end products of lysosomal breakdown (that is, resistant particles of iron). Scale bar, 1  $\mu$ m. (d) Magnified view of lysed bacterial whorls in the host cytoplasm; some magnetosomes appear in chains (m), others are in less dense aggregations. Scale bar, 500 nm.

Sulfur was also identified within the inclusions. Osmium in the sample was an artifact of the postfixation procedure.

#### Magnetic contrast in isolated symbionts

AFM topography images showed clumps of cellular material, with a height range of approximately 120 nm (Figure 3a), and MFM imaging revealed clear magnetic contrast in certain regions, measuring <200 nm (Figure 3b). The magnetic contrast remained evident at MFM tip fly heights of 100 nm, which is far beyond the distance over which van der Waals interactions would be significant.

#### Crystalline structure of inclusions

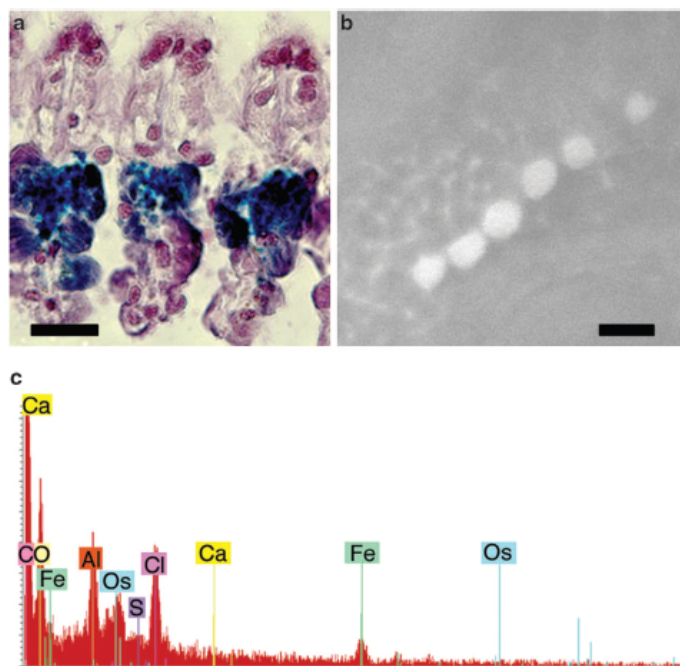
Five diffraction patterns were obtained using SAED, and the following d-spacings were determined: 2.94, 2.5 (two times), 1.56 (three times) and 1.8 Å. Comparisons with reference materials (greigite, magnetite, mackinawite, hematite and pyrite; Lennie *et al.*, 1995; Downs, 2006) indicate that inclusions are most likely a combination of greigite and mackinawite (Supplementary Table 1).

#### Symbiont identification and phylogeny

We amplified a single 16S rRNA sequence from each host specimen, with sequences from each host individual being exactly the same (length = 1430 bp, accession number: KJ658209), and used it to construct a phylogenetic tree (Figure 4). Symbionts belong to the gammaproteobacteria and are closely related to the symbionts of other invertebrates, including *Thyasira flexuosa* (98.5% identity, accession number L01575.1) and the hydrothermal vent tubeworm *Riftia pachyptila* (94% identity), as well as free-living bacteria from marine sediments (Figure 4). The magnetotactic bacterium that is most similar to the *T. cf. gouldi* symbiont (93% similarity) is gammaproteobacterium SS-5 isolated from the Salton Sea (Lefèvre *et al.*, 2012). Other more closely related bacteria are not known to have magnetosomes.

From the same DNA extracts, we amplified the gene for RuBisCO form II (*cbbM*, length = 397 bp, accession number: KJ658208), but could not amplify the gene for form I RuBisCO (*cbbL*). The most closely matched sequence (89% identity) was from a symbiont of a deep-sea tubeworm (*Lamellibranchia* sp., accession no. FM165442). There are no other thyasirid symbiont RuBisCO sequences available in GenBank.

FISH with the symbiont-specific 16S probe resulted in positive labeling of bacteria extracted from sediments collected at the three study sites, at all depth fractions examined. Following extraction of DNA from Bonne Bay sediments and analysis of PCR product sequences, we identified reads matching the known *Thyasira cf. gouldi* 16S sequence



**Figure 2** Iron determination in bacteriocytes and in the magnetosomes of *Thyasira cf. gouldi* symbionts. (a) Transverse section of a gill of *T. cf. gouldi*, stained with prussian blue and hematoxylin. The cytoplasm of bacteriocytes is intensely stained, indicating the presence of ferric iron. Scale bar, 20  $\mu$ m. (b) Backscatter electron image of a symbiont, showing a chain of magnetosomes. Scale bar, 100 nm. (c) Energy dispersive X-ray spectrum after analysis of a particle within the magnetosome chain. Peaks for iron are visible.

with 98–100% similarity in both the samples. This level of specificity allows for natural mutations within the population as well as sequencing errors; other authors have considered that sequences >97% identical belong to the same microbial species (Huber *et al.*, 2007). Reads that aligned represented about 4% of the total data, suggesting that the symbiont represents a small portion of the bacterial community within these sediments. A blastn search revealed that the aligned reads were more similar to the *T. cf. gouldi* sequence than to any sequences available in the NCBI non-redundant database, with the top blast hits being 96% similar and originating from uncultured marine environmental samples. Therefore, there are free-living forms of the *T. cf. gouldi* symbionts in native sediments.

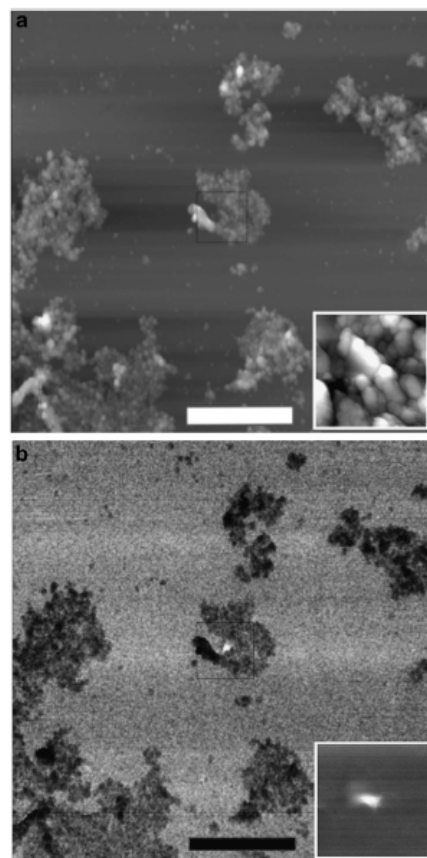
## Discussion

Together, our results provide evidence for the presence of magnetosomes in the symbionts of *Thyasira cf. gouldi*, and allow us to propose a mechanistic pathway for symbiont acquisition in this species. We identified a single bacterial phylotype belonging to the sulfur-oxidizing gammaproteobacteria in *T. cf. gouldi*, as is typical of other

thyasirids and many other chemosymbiotic bivalves (Dubilier *et al.*, 2008; Duperron *et al.*, 2012b). The single bacterial phylotype obtained from DNA extractions of *T. cf. gouldi* gills represents a symbiont and not simply an environmental contaminant trapped on epithelial cells, based on the following evidence: (1) numerous ( $N > 80$ ) symbiotic *T. cf. gouldi* specimens observed using TEM contain morphologically similar bacteria; (2) different host individuals ( $N = 3$  in this study) associate with the same, unique symbiont phylotype, a situation that would be highly unlikely if these bacteria were simply filtered from seawater; and (3) host epithelial cells clearly endocytose these bacteria similar to other bivalves where this process was demonstrated to be a pathway for nutrient transfer (Streams *et al.*, 1997).

TEM observations show that inclusions are common among *Thyasira cf. gouldi* symbionts and fall within the general range of morphologies and sizes (tens of nanometers) reported for magnetosomes (Lefèvre *et al.*, 2013a). The occasional chains of inclusions, along with the demonstration of iron (with magnetic properties) in both inclusions and host bacteriocytes show that those structures are not viruses, but rather magnetosomes. The diffraction patterns obtained from these inclusions suggest that particles are a mixture of the iron sulfides greigite





**Figure 3** Simultaneously acquired AFM and MFM images of isolated *Thyasira cf. gouldi* symbionts. Scale bar, 5  $\mu\text{m}$ . (a) AFM topography image of dried symbionts, where taller features are shown in white. Magnetosomes are not evident. Inset shows AFM image of outlined area. (b) MFM phase-contrast image of the area shown in a at a fly height of 100 nm. White and black regions indicate magnetic interactions between the sample and the magnetic tip. Inset shows MFM image of outlined area.

and its precursor mackinawite as in other marine and estuarine species of magnetotactic bacteria (Pósfai *et al.*, 1998), including gammaproteobacteria (Simmons *et al.*, 2004). Mackinawite is non magnetic and is converted to greigite in magnetotactic bacteria (this transformation was observed after sample preparation and exposure to air; Pósfai *et al.*, 1998), therefore, the presence of both forms of these minerals within a population of bacteria with magnetosomes is not surprising. The presence of those minerals is consistent with the identification of sulfur within inclusions using elemental analysis, and the shape of inclusions resembles that of other described greigite and mackinawite particles (Pósfai *et al.*, 1998; Spring and Bazylinski, 2006). Further, we noted less MFM signal than we expected in our bacterial cell preparations, which

may indicate that an important fraction of the magnetosomes contained mackinawite in our preparations. Recently, a strain of sulfate-reducing magnetotactic deltaproteobacteria (BW-1) was shown to biomineralize both greigite and magnetite, depending on environmental conditions (Lefevre *et al.*, 2011); another freshwater strain of deltaproteobacteria simultaneously produces magnetite and greigite (Wang *et al.*, 2013). We have no evidence for similar physiological capabilities in the symbiont of *T. cf. gouldi*, but given that only five diffraction patterns were obtained herein, we cannot affirm that this species is unable to biomineralize magnetite.

Although the presence of magnetotactic bacteria within a host organism appears counterintuitive as symbionts have no need for magnetotaxis, a consideration of the entire ecological spectrum of these bacteria, along with host behaviors, helps to elucidate their presence in host organisms. The existence of free-living symbionts in sediments from the *Thyasira cf. gouldi* habitat suggests horizontal symbiont transmission in these thyasirids. In their free-living state, the symbionts of *T. cf. gouldi* would benefit from magnetotaxis (or magneto-aerotaxis, Frankel *et al.*, 2006) as a means of tracking OAI in sediments, thereby facilitating sulfur oxidation for chemolithoautotrophy (as in other marine magnetotactic bacteria, Bazylinski and Williams, 2006). However, magnetotaxis would require the presence of flagella, which are not apparent on TEM images of gill-associated symbionts. The *T. cf. gouldi* symbiont is likely able to produce a flagellum when free living, and to lose it once associated with its host: the closely related, facultative symbiont of *Riftia pachyptila* (*Cad. Endoriftia persephone*) contains genes required for a functional flagellum and for chemotaxis, although flagella have never been observed in symbionts (Millikan *et al.*, 1999, Robidart *et al.*, 2008). Proteomic studies suggest physiological differences between host-associated and free-living states of *Cad. Endoriftia persephone* (Markert *et al.*, 2007), and physiological differences probably also exist between free living and associated *T. cf. gouldi* symbionts. In magnetotactic bacteria, the physiological state was shown to affect magnetosome formation (Bazylinski and Williams, 2006).

Thyasirid bioirrigation can produce OAI along burrow walls (Dando *et al.*, 2004; Hakonen *et al.*, 2010), thereby enhancing the concentration of magnetotactic bacteria in near-burrow sediments. *Thyasira cf. gouldi* could collect symbionts from burrow walls on the mucociliary surface of their elongated feet (Allen, 1958). Upon retraction of the foot within the mantle cavity, symbionts trapped in mucus could collect on the gill. Once associated with host cells, symbionts would have no requirement for a flagellum or a functional magnetosome, as the host acquires oxygen and sulfide through bioirrigation and sulfide-mining behaviors (Dufour



**Figure 4** Bayesian phylogenetic tree constructed using 16S rRNA gene sequences (1627 positions in the alignment, see text for method used), showing the relationships between the *Thyasira* cf. *gouldi* symbiont, other gammaproteobacteria (symbiotic or free living) and known magnetotactic bacteria (in bold). Numbers represent posterior probabilities calculated in MrBayes (v. 3.2.2). Accession numbers are in parentheses.

and Felbeck, 2003; Dando *et al.*, 2004). Interestingly, the biomagnetic crystals are rarely organized in a chain in *T. cf. gouldi* symbionts, suggesting that proteins responsible for chain formation are not maintained in *hospite*. Other magnetotactic bacteria with clusters of magnetosome crystals rather than chains retain magnetotactic abilities (Cox *et al.*, 2002; Zhang *et al.*, 2013); however, the crystals in those species are aggregated at one end of the bacterial cell and not scattered throughout the cytoplasm as in *T. cf. gouldi* symbionts. Further, the apparent presence of both mackinawite and greigite (with the former possibly predominating) in the gill-associated symbionts of *T. cf. gouldi* begs the question of whether mechanisms for transforming mackinawite to greigite remain functional in associated symbionts. The few *T. cf. gouldi* symbionts with intact chains of magnetosomes may be recently acquired individuals, suggesting that symbiont uptake in thysirids can occur more than once in their adult life, as in lucinids (Gros *et al.*, 2012).

The whorls in the bacteriocytes of *Thyasira* cf. *gouldi* indicate that host cells can engulf and digest symbionts, a likely means of nutrient uptake in thyasirids (Southward, 1986; Dufour, 2005). Through this process, magnetic particles appear

resistant to host degradation, concentrating in whorls and in the cytoplasm of epithelial cells. However, particles within host cytoplasm are slightly larger and appear more diffuse than those within intact bacteria. Similarly, ciliates having engulfed magnetotactic bacteria accumulated enlarged magnetosomes in their cytoplasm (Martins *et al.*, 2007), and the size increase was interpreted as showing magnetosome dissolution. Occasionally, host gills contain such dense concentrations of particles that they appear black; such gills stained intensely with prussian blue and were therefore iron-rich. *T. cf. gouldi* may eliminate magnetic particles through the renewal of epithelial cells, an active process in bivalve gills (Hanselmann *et al.*, 2000).

Although much remains to be learned about the phylogeny and metabolic capabilities of *Thyasira* cf. *gouldi* (and other thyasirid) symbionts, 16S rRNA sequencing supports their phylogenetic placement among other sulfur-oxidizing gammaproteobacteria, and the amplification of type II RuBisCO provides evidence for autotrophic metabolism in these bacteria. The symbionts of *T. cf. gouldi* represent one of the few described magnetotactic gammaproteobacteria (Simmons *et al.*, 2004; Lefevre *et al.*, 2012; Wang *et al.*, 2013). Genomic studies have shown that



genes responsible for magnetosome formation are often organized within 'magnetosome islands' that contain mobile elements (Ullrich *et al.*, 2005; Jogler *et al.*, 2009), and recent work suggests a monophyletic origin for magnetotaxis within the proteobacteria (Lefèvre *et al.*, 2013b). Inclusions resembling magnetosomes, but interpreted as viruses, have been noted in other thyasirid symbionts, notably in 'symbiont of *Thyasira flexuosa* 1' from the Mediterranean, in Figure 4; (Brissac *et al.*, 2011), in symbionts of *T. gouldi* from Scotland (Southward and Southward, 1991) and of *T. flexuosa* from Long Beach, USA (Dufour, 2005) and Brest, France (J. Laurich, personal communication). Further phylogenetic and ecological studies of thyasirids and their symbionts should clarify why hosts from such different locations associate with closely related, magnetosome-forming symbionts.

The discovery of magnetotactic bacteria as symbionts of bivalves underscores their vast adaptability (Lefèvre and Bazylinski, 2013) and provides new opportunities to study magnetosome formation in an ecological context. The association of such bacteria with thyasirids is likely facilitated by the host's sulfide mining and bioirrigation activities, which produce OAI along burrow linings. In thyasirids, the combination of host and symbiont behaviors appears to have been key to chemosymbiosis establishment, enhancing the success of this family in a variety of sedimentary environments.

## Conflict of Interest

The authors declare no conflict of interest.

## Acknowledgements

R Hooper helped with specimen collection. M Shaffer, MUN Micro Analysis Facility, provided assistance with ESEM and A Korinek from the Canadian Centre for Electron Microscopy, McMaster University, performed SAED. H Fal and R Zadra assisted with histology and A Bullen performed RubisCO amplification. A Lang, Y Fu, H Munro, L Peña-Castillo helped with the analysis of environmental 16S sequences. Funding was obtained through NSERC Discovery Grant 386087-2010 (SCD), NSERC post-graduate scholarship (JRL), RDC Ignite R&D Grant 5404.1126.101 and RDC Ocean Industries Student Research Award (RTB).

## References

- Allen JA. (1958). On the basic form and adaptations to habitat in the Lucinacea (Eulamellibranchia). *Philos Trans Roy Soc B* **241**: 421–484.
- Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ. (1990). Basic local alignment search tool. *J Mol Biol* **215**: 403–410.
- Batstone RT, Laurich JR, Salvo F, Dufour SC. (2014). Divergent chemosymbiosis-related characters in

- Thyasira* cf. *gouldi* (Bivalvia: *Thyasiridae*). *PLoS ONE* **9**: e92856.
- Bazylinski DA, Williams TJ. (2006). Ecophysiology of magnetotactic bacteria. *Microbiol Monogr* **3**: 37–75.
- Brissac T, Rodrigues CF, Gros O, Duperron S. (2011). Characterization of bacterial symbioses in *Myrtea* sp. (Bivalvia: *Lucinidae*) and *Thyasira* sp. (Bivalvia: *Thyasiridae*) from a cold seep in the eastern Mediterranean. *Mar Ecol* **32**: 198–210.
- Cox BL, Popa R, Bazylinski DA, Lanoil B, Douglas S, Belz A *et al.* (2002). Organization and elemental analysis of P-, S-, and Fe-rich inclusions in a population of freshwater magnetococci. *Geomicrobiol J* **19**: 387–406.
- Dando PR, Spiro B. (1993). Varying nutritional dependence of the thyasirid bivalves *Thyasira sarsi* and *T. equalis* on chemoautotrophic symbiotic bacteria, demonstrated by isotope ratios of tissue carbon and shell carbonate. *Mar Ecol Prog Ser* **92**: 151–158.
- Dando PR, Southward AJ, Southward EC. (2004). Rates of sediment sulphide oxidation by the bivalve mollusc *Thyasira sarsi*. *Mar Ecol Prog Ser* **280**: 181–187.
- Distel DL, Felbeck H. (1988). Pathways of inorganic carbon fixation in the endosymbiont-bearing lucinid clam *Lucinoma aequizonata*. part 1. purification and characterization of the endosymbiotic bacteria. *J Exp Zool* **247**: 1–10.
- Downs RT. (2006). The RRUFF Project: an integrated study of the chemistry, crystallography, Raman and infrared spectroscopy of minerals. 19th General Meeting of the International Mineralogical Association, Kobe, Japan.
- Dubilier N, Bergin C, Lott C. (2008). Symbiotic diversity in marine animals: the art of harnessing chemosynthesis. *Nat Rev Microbiol* **6**: 725–740.
- Dufour SC. (2005). Gill anatomy and the evolution of symbiosis in the bivalve family *Thyasiridae*. *Biol Bull* **208**: 200–212.
- Dufour SC, Felbeck H. (2003). Sulphide mining by the superextensible foot of symbiotic thyasirid bivalves. *Nature* **426**: 65–67.
- Duperron S, Rodrigues CF, Léger N, Szafranski K, Decker C, Olu K *et al.* (2012a). Diversity of symbioses between chemosynthetic bacteria and metazoans at the Guinea cold seep site (Gulf of Guinea, West Africa). *Microbiologyopen* **1**: 467–480.
- Duperron S, Gaudron SM, Rodrigues CF, Cunha MR, Decker C, Olu K. (2012b). An overview of chemosynthetic symbioses in bivalves from the North Atlantic and Mediterranean Sea. *Biogeosciences Discuss* **9**: 16815–16875.
- Elsaied H, Naganuma T. (2001). Phylogenetic diversity of ribulose-1,5-bisphosphate carboxylase/oxygenase large-subunit genes from deep-sea microorganisms. *Appl Environ Microbiol* **67**: 1751–1765.
- Frankel RB, Williams TJ, Bazylinski DA. (2006). Magneto-aerotaxis. *Microbiol Monogr* **3**: 1–24.
- Gros O, Liberge M, Heddi A, Khatchadourian C, Felbeck H. (2003). Detection of the free-living forms of sulfide-oxidizing gill endosymbionts in the lucinid habitat (*Thalassia testudinum* environment). *Appl Environ Microbiol* **69**: 6264–6267.
- Gros O, Elisabeth NH, Gustave SDD, Caro A, Dubilier N. (2012). Plasticity of symbiont acquisition throughout the life cycle of the shallow-water tropical lucinid *Codakia orbiculata* (Mollusca: Bivalvia). *Environ Microbiol* **14**: 1584–1595.

- Hakonen A, Hulth S, Dufour S. (2010). Analytical performance during ratiometric long-term imaging of pH in bioturbated sediments. *Talanta* **81**: 1393–1401.
- Hanselmann R, Smolowitz R, Gibson D. (2000). Identification of proliferating cells in hard clams. *Biol Bull* **199**: 199–200.
- Huber JA, Welch DBM, Morrison HG, Huse SM, Neal PR, Butterfield DA et al. (2007). Microbial population structures in the deep marine biosphere. *Science* **381**: 97–100.
- Huelsenbeck JP, Ronquist F. (2001). MRBAYES: Bayesian inference of phylogenetic trees. *Bioinformatics* **17**: 754–755.
- Jogler C, Kube M, Schübbe S, Ullrich S, Teeling H, Bazylnski DA et al. (2009). Comparative analysis of magnetosome gene clusters in magnetotactic bacteria provides further evidence for horizontal gene transfer. *Environ Microbiol* **11**: 1267–1277.
- Lane DJ. (1991). 16S/23S rRNA sequencing. In: Stackebrandt E, Goodfellow M (eds) *Nucleic Acid Techniques in Bacterial Systematics*. Wiley and Sons: New York, pp 115–175.
- Lefèvre CT, Bazylnski DA. (2013). Ecology, diversity, and evolution of magnetotactic bacteria. *Microbiol Mol Biol Rev* **77**: 497–526.
- Lefèvre CT, Menguy N, Abreu F, Lins U, Pósfai M, Prozorov T et al. (2011). A cultured greigite-producing magnetotactic bacterium in a novel group of sulfate-reducing bacteria. *Science* **334**: 1720–1723.
- Lefèvre CT, Vioria N, Schmidt ML, Pósfai M, Frankel RB, Bazylnski DA. (2012). Novel magnetite-producing magnetotactic bacteria belonging to the Gammaproteobacteria. *ISME J* **6**: 440–450.
- Lefèvre CT, Trubitsyn D, Abreu F, Kolinko S, Jogler C, Almeida LGP et al. (2013a). Comparative genomic analysis of magnetotactic bacteria from the Deltaproteobacteria provides new insights into magnetite and greigite magnetosome genes required for magnetotaxis. *Environ Microbiol* **15**: 2712–2735.
- Lefèvre CT, Trubitsyn D, Abreu F, Kolinko S, Almeida LGP, Vasconcelos ATR et al. (2013b). Monophyletic origin of magnetotaxis and the first magnetosomes. *Environ Microbiol* **15**: 2267–2274.
- Lennie AR, Redfern SAT, Schofield PF, Vaughan DJ. (1995). Synthesis and Rietveld crystal structure refinement of mackinawite, tetragonal FeS. *Mineral Mag* **59**: 677–683.
- Manz W, Amman R, Ludwig W, Wagner M, Schleifer K-H. (1992). Phylogenetic oligodeoxynucleotide probes for the major subclasses of proteobacteria: problems and solutions. *Syst Appl Microbiol* **15**: 593–600.
- Markert S, Arndt C, Felbeck H, Becher D, Sievert SM, Hügler M et al. (2007). Physiological proteomics of the uncultured endosymbiont of *Riftia pachyptila*. *Science* **315**: 247–250.
- Martins JL, Silveira TS, Abreu F, Silva KT, Silva-Neto ID, Lins U. (2007). Grazing protozoa and magnetosome dissolution in magnetotactic bacteria. *Environ Microbiol* **9**: 2775–2781.
- Millikan DS, Felbeck H, Stein JS. (1999). Identification and characterization of a flagellin gene from the endosymbiont of the hydrothermal vent tubeworm *Riftia pachyptila*. *Appl Environ Microbiol* **65**: 3129–3133.
- Pósfai M, Buseck PR, Bazylnski DA, Frankel RB. (1998). Iron sulfides from magnetotactic bacteria: structure, composition, and phase transitions. *Am Mineral* **83**: 1469–1481.
- Proksh RB, Schäffer TE, Moskowitz BM, Dahlberg ED, Bazylnski DA, Frankel RB. (1995). Magnetic force microscopy of the submicron magnetic assembly in a magnetotactic bacterium. *Appl Phys Lett* **66**: 2582–2584.
- Robidart JC, Bench SR, Feldman RA, Novoradovsky A, Podell SB, Gaasterland T et al. (2008). Metabolic versatility of the *Riftia pachyptila* endosymbiont revealed through metagenomics. *Environ Microbiol* **10**: 727–737.
- Robinson JT, Thorvaldsdóttir H, Winckler W, Guttman M, Lander ES, Getz G et al. (2011). Integrative genomics viewer. *Nat Biotechnol* **29**: 24–26.
- Sheehan DC, Hrapchak BB. (1980). *Theory and Practice of Histotechnology*, 2nd edition Mosby: St. Louis.
- Simmons SL, Sievert SM, Frankel RB, Bazylnski DA, Edwards KJ. (2004). Spatiotemporal distribution of marine magnetotactic bacteria in a seasonally stratified coastal salt pond. *Appl Environ Microbiol* **70**: 6230–6239.
- Spring S, Bazylnski DA. (2006). Magnetotactic bacteria. *Prokaryotes* **2**: 842–862.
- Southward EC. (1986). Gill symbionts in thiasirids and other bivalve molluscs. *J Mar Biol Assoc UK* **66**: 889–914.
- Southward EC, Southward AJ. (1991). Virus-like particles in bacteria symbiotic in bivalve gills. *J Mar Biol Ass UK* **71**: 37–45.
- Streams ME, Fisher CR, Fiala-Médioni A. (1997). Methanotrophic symbiont location and fate of carbon incorporated from methane in a hydrocarbon seep mussel. *Mar Biol* **129**: 465–476.
- Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. (2011). MEGA5: Molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol* **28**: 2731–2739.
- Ullrich S, Kube M, Schübbe S, Reinhardt R, Schüller D. (2005). A hypervariable 130-kilobase genomic region of *Magnetospirillum gryphiswaldense* comprises a magnetosome island which undergoes frequent rearrangements during stationary growth. *J Bacteriol* **187**: 7176–7184.
- Wang Y, Lin W, Pan Y. (2013). High diversity of magnetotactic deltaproteobacteria in a freshwater niche. *Appl Environ Microbiol* **79**: 2813–2817.
- Wolfgang L, Strunk O, Westram R, Richter L, Meier H, Yadukumar AB et al. (2004). Arb: a software environment for sequence data. *Nucleic Acids Res* **32**: 1363–1371.
- Zhang WY, Zhou K, Pan HM, Du HJ, Chen YR, Zhang R et al. (2013). Novel rod-shaped magnetotactic bacteria belonging to Alphaproteobacteria. *Appl Environ Microbiol* **79**: 3137–3140.

Supplementary Information accompanies this paper on The ISME Journal website (<http://www.nature.com/ismej>)